CANCER RESEARCH

A MONTHLY JOURNAL OF ARTICLES AND ABSTRACTS REPORTING CANCER RESEARCH

VOLUME 2 APRIL, 1942 NUMBER 4

The Spectrographic Analysis of Carcinogenic Hydrocarbons and Metabolites

I. Introduction*

R. Norman Jones, Ph.D.

(From the Converse Memorial Laboratory, Harvard University, Cambridge, Mass.)

(Received for publication January 7, 1942)

The quantitative analysis of carcinogenic hydrocarbons and derivatives in tissue extracts by ultraviolet spectrophotometry has not been developed to any considerable extent, although both Chalmers (5) and Lorenz and Shear (21) demonstrated several years ago that such analysis is practicable. Polynuclear aromatic hydrocarbons, including alkyl and alicyclic derivatives, possess intense and very characteristic spectra which have been used frequently to establish the structure of synthetic compounds (11-14). The observation of Shear (25) that a tumor can be initiated in a mouse by as little as 0.4 μgm. of 1,2,5,6-dibenzanthracene dissolved in cholesterol tended to discourage further investigations along these lines for some time, as such quantities are close to the limit of sensitivity of spectrographic analysis even under optimum conditions, and it seemed improbable that spectrometric studies could contribute much to an understanding of the mechanism of tumor initiation by carcinogens if such minute quantities of hydrocarbon are instrumental in initiating the proliferation of cancerous cells.

Recently considerable interest has been shown in the detoxification processes by which hydrocarbon carcinogens are excreted (1, 2, 4, 6, 8) and which may play a role in the removal of the hydrocarbon from the site of injection. In this type of investigation quantities of the order of milligrams of hydrocarbon are involved, and, provided certain technical difficulties can be overcome, spectrophotometric analyses of hydrocarbons in tissue extracts and excreta extracts might yield valuable information concerning the mechanism of detoxification and enable the influence of dietary and other factors on the rate and mode of excretion to be studied quantitatively.

* This investigation was aided by a grant from The International Cancer Research Foundation.

TECHNIC OF SPECTROPHOTOMETRIC ANALYSIS

Most methods of quantitative spectrographic analysis involve the use of a rotating sector or some equivalent optical device by which the ratio of the intensity of monochromatic light I transmitted by a given length of the solution is compared with that transmitted by a cell of the same length containing the solvent only, I_0. This comparison is made over a range of wave lengths. The apparatus used in this laboratory has been described previously (16).

The molecular extinction coefficient ϵ is defined by the equation

$$\epsilon = \frac{1}{c \cdot l} \cdot \log \frac{I_0}{I}$$

where l is the cell length in cm., c the concentration of the absorbing solute in moles per litre, and $\log I_0/I$, the density. Where the molecular weight of the solute is not known or where the solute is not a pure substance, ϵ cannot be evaluated and in such cases it is convenient to use the function $E^{1\%}_{1\text{ cm.}}$ which is defined as

$$E^{1\%}_{1\text{ cm.}} = \frac{1}{w \cdot l} \cdot \log \frac{I_0}{I}$$

where w is the concentration of the solute in gm. per 100 ml. of solution.[1] It will be evident that in the case of a pure absorbing solute

$$E^{1\%}_{1\text{ cm.}} = \frac{10\epsilon}{m}$$

where m is the molecular weight of the solute.

If the solute is a tissue extract containing non-absorbing material as well as absorbing constituents, the observed value of $E^{1\%}_{1\text{ cm.}}$ at a given wave length

[1] The symbols and nomenclature used here have recently been standardized by a joint committee of the *Journal of Biological Chemistry* and the Optical Society of America.

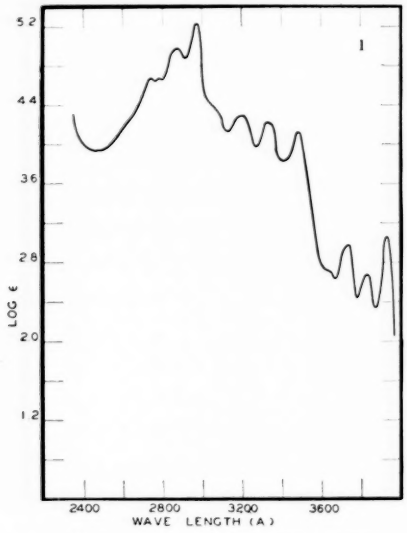


FIG. 1.—1,2,5,6-Dibenzanthracene (solvent ethanol).

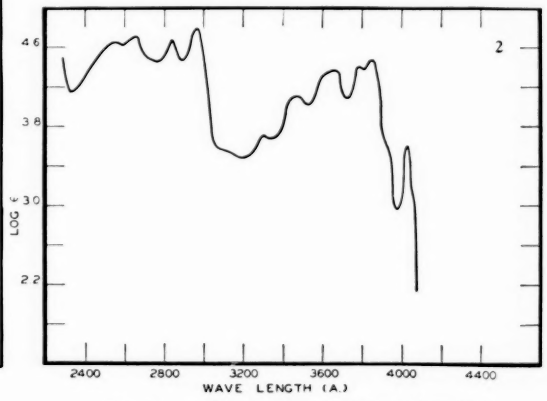


FIG. 2.—3,4-Benzpyrene (solvent ethanol).

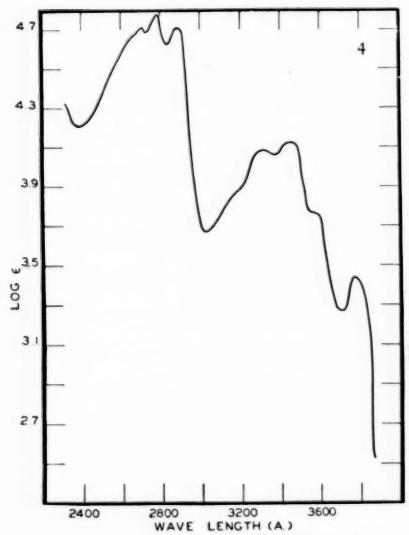


FIG. 4.—4,9-Dimethyl-5,6-benzthiophanthrene (solvent ethanol).

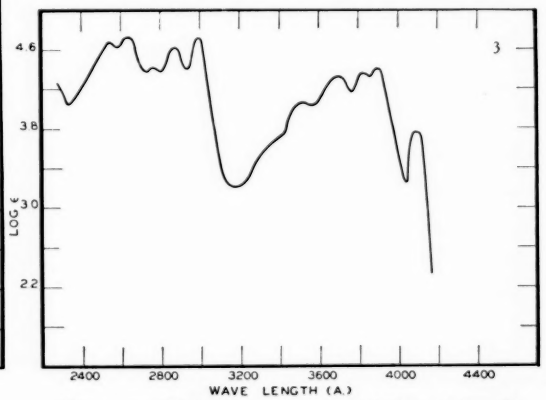


FIG. 3.—2-Methyl-3,4-benzpyrene (solvent ethanol).

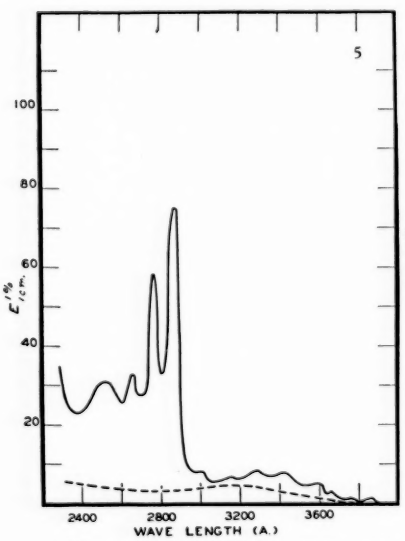


FIG. 5.—Mouse liver extract, nonsaponifiable fraction. *A*, continuous line ————, with 1,2-benzanthracene added. *B*, dash line – – – – –, control.

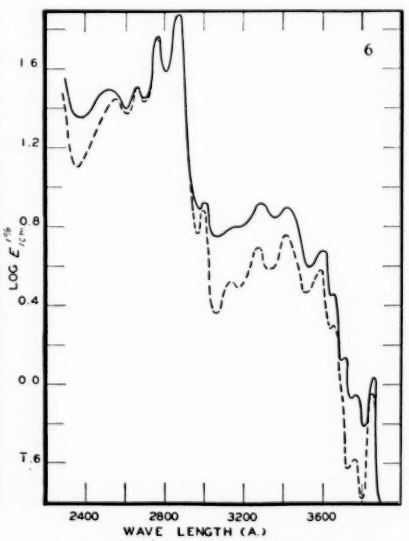


FIG. 6.—*A*, continuous line ————, mouse liver extract, nonsaponifiable fraction with added 1,2-benzanthracene. *B*, dash line – – – – –, master curve of 1,2-benzanthracene.

will be less than that for the pure absorbing substance and the percentage concentration (c_1) of the absorbing constituent in the solute will be given by the equation

$$c_1 = \frac{E^{1\%}_{1\text{ cm.}_{\text{observed}}} \times 100}{E^{1\%}_{1\text{ cm.}_{\text{ pure solute}}}} \%$$

1,2,5,6-dibenzanthracene, 3,4-benzpyrene, 3,4-benzphenanthrene, chrysene, and their respective alkyl and alicyclic derivatives.

The absorption spectrum of 1,2-benzanthracene in ethanol was measured first by Capper and Marsh (3) and later by Mayneord and Roe (22) who have also

TABLE I: REFERENCES TO ABSORPTION SPECTRA OF CARCINOGENIC HYDROCARBONS AND RELATED COMPOUNDS

Compound	Solvent	References
1,2-Benzanthracene	Ethanol	3, 16, 22
1′-Methyl-1,2-benzanthracene	Ethanol	16
4-Methyl-1,2-benzanthracene	Ethanol	16
5-Methyl-1,2-benzanthracene	Ethanol	16
6-Methyl-1,2-benzanthracene	Ethanol	22
7-Methyl-1,2-benzanthracene	Ethanol	22
8-Methyl-1,2-benzanthracene	Ethanol	16
9-Methyl-1,2-benzanthracene	Ethanol	16
10-Methyl-1,2-benzanthracene	Ethanol	16
6-Isopropyl-1,2-benzanthracene	Ethanol	23
10-Isopropyl-1,2-benzanthracene	Ethanol	22
5,8-Dimethyl-1,2-benzanthracene	Ethanol	16
5,10-Dimethyl-1,2-benzanthracene	Ethanol	16
6,7-Dimethyl-1,2-benzanthracene	Ethanol	22
8,10-Dimethyl-1,2-benzanthracene	Ethanol	16
9,10-Dimethyl-1,2-benzanthracene	Ethanol	16
3′-Isopropyl-10-methyl-1,2-benzanthracene	Ethanol	17
5,6-Cyclopenteno-1,2-benzanthracene	Ethanol	22
6,7-Cyclopenteno-1,2-benzanthracene	Ethanol	22
Cholanthrene	Ethanol	12, 23
6-Methylcholanthrene *	Ethanol	17
20-Methylcholanthrene *	Ethanol	22
6,20-Dimethylcholanthrene *	Ethanol	17
6,22-Dimethylcholanthrene *	Ethanol	17
1′,9-Methylene-1,2-benzanthracene	Ethanol	16
10-Methyl-1′,9-methylene-1,2-benzanthracene	Ethanol	17
1,2,5,6-Dibenzanthracene	Benzene	7
1,2,5,6-Dibenzanthracene	Ethanol	5, 22 This paper, Fig. 1
1,2,5,6-Dibenzanthracene	Chloroform	5
1,2,5,6-Dibenzanthracene	Ether	21
2′-Methyl-1,2,5,6-dibenzanthracene †	Ethanol	2
3′-Methyl-1,2,5,6-dibenzanthracene †	Ethanol	2
9,10-Dimethyl-1,2,5,6-dibenzanthracene	Ethanol	22
Chrysene	Ethanol	22
5-Methylchrysene	Ethanol	18
4,5-Dimethylchrysene	Ethanol	18
5,6-Dimethylchrysene	Ethanol	18
4,5-Methylenechrysene	Ethanol	18
3,4-Benzpyrene	Ethanol	22 This paper, Fig. 2
2-Methyl-3,4-benzpyrene	Ethanol	This paper, Fig. 3
8(or 9?)-Methyl-3,4-benzpyrene	Ethanol	24
3,4-Benzphenanthrene	Ethanol	23
4,9-Dimethyl-5,6-benzthiophanthrene	Ethanol	This paper, Fig. 4
4′,8′-Dihydroxy-1,2,5,6-dibenzanthracene	Ethanol	4
Isomeric dihydroxy-1,2,5,6-dibenzanthracene from rabbit urine	Ethanol	2

* Ring numbering according to Fieser's system (see Reference 12).
† Described but no numerical data given.

ABSORPTION SPECTRA OF PURE HYDROCARBONS

Accurate data on the absorption spectra of the pure compounds is a primary requisite for the analysis of carcinogenic hydrocarbons and related substances in tissue extracts. In Table I, references have been summarized relating to the spectra of 1,2-benzanthracene, published accurate spectrographic curves of numerous polynuclear aromatic hydrocarbons (23). Jones (16) has also measured the absorption spectrum of 1,2-benzanthracene in ethanol as well as the spectra of several derivatives (17). Reference should be made to his paper for a comparison of his intensity data for

1,2-benzanthracene with those obtained by Mayneord and Roe.

Clar (7) recorded the spectrum of 1,2,5,6-dibenzanthracene in benzene solution, an unfortunate choice of solvent as benzene has strong absorption at wave lengths less than 2,700 A. Clar's curve is not plotted in a manner which is readily convertible to molecular extinction coefficients. The curve published by Chalmers (5) illustrates only a small section of the spectrum in the region between 2,700 and 3,100 A., the intensities being given for a 0.0005 per cent solution and not calculated as ϵ or $E^{1\%}_{1\text{ cm.}}$. A complete curve for an ethereal solution is given by Lorenz and Shear (21) and for an alcoholic solution by Mayneord and Roe (22). In neither case is the curve accompanied by a table of the intensity values of the maxima, and significant errors may be introduced in interpolation. The

Table II: Absorption Spectrum of 1,2,5,6-Dibenzanthracene; Wave Lengths and Intensities of the Maxima

Jones * (ethanol)		Mayneord and Roe (ethanol)		Lorenz and Shear (ether)	
λ	log ε	λ	log ε	λ †	log ε
2740	4.68	2750	4.7	....	...
2780	4.68	2780	4.7	....	...
2875	4.98	2875	5.0	2880	5.3
2970	5.24	2970	5.2	2970	5.8
3200	4.29	3200	4.3	3205	4.3
3330	4.23	3330	4.2	3355	4.2
3485	4.13	3490	4.2	3500	4.1
3650	2.70	3630	2.7	....	...
3740	2.98	3730	3.0	3745	3.2
3835	2.68	3830	2.6	3850	2.5
3930	3.06	3930	3.1	3970	3.2

* m.p. = 265-266.5° (corr.).
† Converted from wave numbers.

absorption curve for 1,2,5,6-dibenzanthracene in absolute ethanol, as determined by the present author, is given in Fig. 1, and in Table II the intensities of the maxima are compared with the values interpolated from the curves of Mayneord and Roe (22, 23) and of Lorenz and Shear (21). The curve of the latter authors is more intense than the other two in the region of the highest intensity maxima. This difference may be accounted for in part by the change of solvent, and it should be emphasized that such a change will alter the intensities of the maxima in almost all cases by a significant amount; in solvents of low dielectric constant such as hexane and ether, higher intensities and more resolution of the structure of the bands are observed than in more polar solvents such as ethanol, methanol, or water.

The intensities of the maxima are influenced to some extent by the resolving power of the spectrograph. A slit-width of 0.12 mm. was used in all measurements made by the author.

The spectrum of 3,4-benzpyrene in ethanol has been measured both by Mayneord and Roe and by the present author, and the data are compared in Table III. The spectrum of 2-methyl-3,4-benzpyrene, the high carcinogenic activity of which has recently been demonstrated [2] and of the carcinogenic sulfur-containing substance, 4,9-dimethyl-5,6-benzthiophanthrene are given in Figs. 3 and 4 and Tables IV and V.

Table III: Absorption Spectrum of 3,4-Benzpyrene; Wave Lengths and Intensities of the Maxima

Jones * (ethanol)		Mayneord and Roe † (ethanol)	
λ	log ε	λ	log ε
2545	4.66	2540	4.6
2660	4.70	2650	4.7
....	...	2750	4.45
2840	4.68	2840	4.65
2965	4.78	2960	4.8
3300	3.71	3280	3.7
3465	4.12	3460	4.1
3655	4.38	3640	4.4
3785	4.41	....	...
3850	4.48	3840	4.4
4030	3.62	4030	3.6

* m.p. = 179-179.5° (corr.).
† Referred to by Mayneord and Roe as 1,2-benzpyrene.

Table IV: Absorption Spectrum of 2-Methyl-3,4-Benzpyrene *; Wave Lengths and Intensities of the Maxima

(ethanol)			
λ	log ε	λ	log ε
2540	4.68	3520	4.06
2640	4.74	3695	4.33
2760	4.42	3825	4.35
2870	4.62	3895	4.40
2995	4.73	4090	3.76

* m.p. = 165-167° (corr.).

Table V: Absorption Spectrum of 4,9-Dimethyl-5,6-Benzthiophanthrene *; Wave Lengths and Intensities of the Maxima

(ethanol)			
λ	log ε	λ	log ε
2705	4.71	3455	4.15
2790	4.78	3570	3.76
2890	4.71	3770	3.44
3305	4.08		

* m.p. = 158-159° (corr.).

Effect of Alkyl and Alicyclic Substituents

Investigations, chiefly in the 1,2-benzanthracene series (16, 17, 22, 23), have shown that an alkyl group or an alicyclic ring (such as that present in cholanthrene) does not alter the shape of the absorption curve significantly nor does it change the molar extinction coefficients of most of the maxima very appreciably; alkyl and alicyclic derivatives of polynuclear aromatic

[2] Private communication from Dr. C. E. Dunlap.

hydrocarbons can be related structurally to the parent unsubstituted hydrocarbon by comparison of the absorption spectra (12-14). Certain of the maxima may, however, be changed quite considerably in intensity by the introduction of an alkyl substituent and it would seem inadvisable to attempt to analyze quantitatively solutions of alkyl or alicyclic derivatives of these hydrocarbons unless the absorption curve of the particular alkyl or alicyclic derivative is available for comparison.

Cholanthrene, 20-methylcholanthrene, 2-methyl-3,4-benzpyrene, and 9,10-dimethyl-1,2-benzanthracene are the most potent carcinogenic hydrocarbons of this type and the spectra of all of these have been recorded (Table I).

Quantitative Analysis of Solutions of Pure Hydrocarbons

The accuracy with which pure hydrocarbons can be determined is limited by the accuracy with which the extinction coefficient can be determined, which, in turn, is limited by the accuracy of measurement of the density (log I_0/I). This has been discussed for photographic methods using a sector or Hilger-Spekker photometer by Holiday (15) who observes that "it may therefore be conservatively stated that the error in the visual match point method of spectrography is less than ± 0.055 E for a single matching" (E is here equivalent to the density). Twyman and Allsopp (26) consider that the *least* error in density measurement likely to be achieved in practice is ± 0.005 in the density reading. It is evident that as percentage errors these will be less at high densities than at low ones and quantitative measurements should always be made at densities greater than unity. It is not easy to match intensities nearer than 0.05 density units and at a density of 1.5 this corresponds to an error of ± 3.3 per cent. With photoelectric instruments greater accuracy in determining the density is possible but errors from other sources, such as uncertainty in cell length and cell alignment and effects of stray light, soon manifest themselves as factors limiting the significance of the measurements. Some experimental results obtained in these laboratories illustrating the accuracy obtainable in practice are shown in Table VI.

The limiting concentration which can be analyzed, assuming a solution of the pure hydrocarbon not contaminated with absorbing impurities, will be determined by the cell length and the molar extinction coefficient of the most intense maximum of the absorption curve. Of more interest than the limiting concentration is the limiting absolute quantity which can be determined and this will depend in addition on the ratio of the length to volume of the available cells. To fill the 1 cm. cell used in these laboratories, 2 ml. of solution are required while only 5.5 ml. are required for the 4 cm. cell; thus greater sensitivity is obtained by using a more dilute solution in the longer cell. Assuming that the concentration is based on a density measurement of 0.50 in a 4 cm. cell using 5.5 ml. of solution, the minimum quantities of several hydrocarbons which can be determined spectrographically are given in Table VII.

Analysis of Tissue Extracts

It is seldom that sensitivity or accuracy of the order discussed in the previous paragraph will be realized in the analysis of tissue extracts. Such extracts almost invariably contain other absorbing substances, in addition to the hydrocarbon, which give rise to "background" absorption; usually this is devoid of fine structure but often has a broad, flat maximum. The effect of this background absorption will depend upon the ratio of its intensity to that of the hydrocarbon and may vary from a negligible quantity to an amount sufficient to obscure the hydrocarbon curve completely. Examples of spectra showing various degrees of background absorption will be found in the succeeding papers (19, 20).

By suitable processing of the extracts, the background frequently may be reduced to a level which makes the analytical results significant. Lorenz and Shear (21) describe a method for the preparation of extracts of 1,2,5,6-dibenzanthracene from tumor tissue, based on a series of solvent partitions. Generally speaking,

Table VI: Spectrographic Analysis of Solutions of Pure Carcinogens and Related Substances

Substance	Added	Found	Error, in per cent
Anthracene	0.804γ	0.810γ	0.8
20-Methylcholanthrene	38.6	38.5	0.3
20-Methylcholanthrene	245	254	3.7
1,2-Benzanthryl-10-carbamidoacetic acid	67.5	65.5	3.0
	43.0	43.0	0.0
	39.8	40.8	2.5
	5.7	5.7	0.0
1,2-Benzanthryl-3-carbamidoacetic acid	79.0	82.6	4.5

Table VII: Minimum Quantities of Hydrocarbons Which Can Be Analyzed Spectrographically in a 4 cm. Cell Containing 5.5 ml. of Solution

Hydrocarbon	ε maximum	Quantity
1,2-Benzanthracene	89,130	1.75γ
1,2,5,6-Dibenzanthracene	173,800	1.09
3,4-Benzpyrene	60,260	2.90
20-Methylcholanthrene	89,130	2.07
4,9-Dimethyl-5,6-benzthiophanthrene	60,260	3.00

methods of purification based upon chemical separation of the interfering substances from the hydrocarbon are preferable to methods based upon differences in physical properties. This may be illustrated in one case by the example given in Table VIII where the results of some experiments are recorded which were undertaken with the object of freeing extracts containing methylcholanthrene from hydroxyl-containing constituents. Two methods were investigated, a physical method based upon the preferential adsorption of the hydroxylic compounds on alumina from benzene solution and a chemical method based upon the reaction of the hydroxylic compounds with succinic anhydride to form an alkali-soluble half ester. While recovery of the 20-methylcholanthrene from the succinate reaction mixture was quantitative, an appreciable loss accompanied the chromatographic adsorption process.

Although complete removal of background absorption often may not be realizable, valuable data may still be obtained from spectrophotometric analyses of tissue extracts, provided it can be established that the relative contribution of the extraneous absorbing material is small compared with that of the hydrocarbon. A similar problem is encountered in the spectrographic analysis of vitamin A where, in spite of uncertainty introduced by this cause, the absorption intensity at 3,280 A. is still regarded as a more accurate indication of the vitamin A content of fish oils and similar products than colorimetric determination with the Carr Price reagent (27).

TABLE VIII: EFFECT OF PROCESSING ON THE SPECTROGRAPHIC ANALYSIS OF 20-METHYLCHOLANTHRENE

	Added	Found	Error, in per cent
Chromatographed from benzene on alumina	3.32 mgm.	3.08 mgm.	7.2
Refluxed with succinic anhydride in pyridine-dioxane	245γ	247γ	0.82
Refluxed with succinic anhydride in pyridine	245	245	0.0
Refluxed with maleic anhydride in ether	245	231	5.7

To illustrate the effects of background absorption, experiments may be cited in which rat or mouse liver tissue was ground with sand, and 3 to 4 mgm. of 1,2-benzanthracene added to one-half of the liver tissue, the remainder of the tissue being worked up as a control. After dehydration in a vacuum desiccator, the two samples were extracted in Soxhlet extractors with chloroform until the fresh extracts from the sample containing added hydrocarbon were no longer fluorescent (24 hrs.). After removal of the solvent *in vacuo* in a current of nitrogen the extracts were dissolved in 50 ml. of 2 N potassium hydroxide in 90 per cent methanol and refluxed for 2.5 hours. The saponification liquor was diluted with water, extracted with ether and the ether extracts, after removal of the solvent, dissolved in 50 ml. of ethanol, and the spectra determined, the intensity being calculated as $E^{1\%}_{1\text{ cm.}}$ based on the weight of the extract (Fig. 5). In this experiment the absorption spectrum of the control extract may be regarded as the background absorption of the extract containing added 1,2-benzanthracene and by subtracting this background curve a corrected value for the 1,2-benzanthracene content of the extract can be calculated. The results of two such experiments using mouse and rat livers, respectively, are summarized in Table IX. In one case where 3.34 mgm. of hydrocarbon were added to 2 gm. of dried ground rat liver tissue the quantity recovered was 3.58 mgm., neglecting the background absorption, which fell to 3.49 mgm. on making the correction. In this case the position of maximum background absorption at 3,220 A. and the fact that the extract gave a deep blue color with the Carr Price reagent suggested that vitamin A might be the substance contributing most to the background absorption. Treatment of the extract with maleic anhydride in ether removed the background absorption almost completely but also caused some loss of 1,2-benzanthracene. This is shown up in the spectrographic results obtained after treatment with maleic anhydride where the correction for the control is negligible but a significant fall in the estimated 1,2-benzanthracene also has occurred (Table IX).

TABLE IX: EFFECT OF BACKGROUND ABSORPTION ON THE SPECTROGRAPHIC ANALYSIS OF 1,2-BENZANTHRACENE IN RAT AND MOUSE LIVER TISSUE

	Found, mgm.
Experiment 1. 3.34 mgm. added to 2 gm. rat liver	
Uncorrected for background absorption	3.58
Corrected by subtraction of background determined on control experiment	3.49
Same extract after treatment with maleic anhydride in ether (to remove vitamin A)	3.08
Same, corrected for background absorption by control experiment	3.05
Experiment 2. 4.70 mgm. added to 1.58 gm. mouse liver	
Uncorrected for background absorption	4.79
Corrected	4.56

This method of running control spectra is probably the most satisfactory way of correcting for the effect of background absorption; unfortunately, in addition to the fact that it involves a complete duplication of the experimental procedure and is therefore time-consuming, it may not always be reliable since it cannot be known with certainty that the injection of the carcinogen may not bring about secondary changes in metabolism which alter the concentration of the sub-

stances in the extracts which are responsible for the background absorption.

Graphical Estimation of Effects of Background Absorption

An indication of the contribution of the background to the total absorption may be obtained by comparing the shape of the experimentally observed curve with that of the pure hydrocarbon. It is not common to find background absorption of a uniform intensity over the whole spectrum and comparison of the experimental curve with a "master curve" of the hydrocarbon drawn on the same scale [3] will indicate the region in which the background is a minimum as that where the two curves fit most closely; the extent of the deviation of the experimental curve from the master curve will enable a qualitative estimate to be made of the degree to which the extraneous absorption is influencing the spectrum. By calculating the concentration from extinction coefficients in the region of the curve which agrees most closely with the master curve, the effect of the background absorption can be reduced to a minimum. This is illustrated in Fig. 6 where the curve for 1,2-benzanthracene, plotted on the same scale, is superimposed on the experimental curve for a liver extract containing 1,2-benzanthracene. The presence of a maximum in the background absorption near 3,200 A. is evident from the deviation between the two curves in this region. The master curve, plotted on the same scale on transparent paper is placed over the experimental curve and the ordinates adjusted so that it touches the experimental curve where possible, *but at no point lies above it.* At any wave length, the total absorption in the experimental curve will be due to two components, the hydrocarbon (E_h) and the background component (E_b). By this graphical procedure the curves will coincide where E_b is a minimum and, at all wave lengths where E_b is greater, the experimental curve will be more intense, the difference between the curves giving a measure of the magnitude of the background effect. By calculating the concentration of the hydrocarbon from points where the experimental and master curves coincide, the effect of the background is reduced to a minimum. The concentration of hydrocarbon so calculated may be regarded normally as a maximum and the significance which can be attached to the result will depend upon the extent to which the experimental curve deviates from the master curve. Where the two coincide over the greater part of the spectrum, the calculated concentration can be accepted with confidence as accurate. Where the curves differ markedly the results may be in error, as in such cases it is probable that even in the region where there is coincidence considerable enhancement of the intensity due to background may be occurring and the concentration of hydrocarbon so calculated is likely to be high.[4]

[3] As this is purely an empirical method, curves plotted on a logarithmic intensity scale are convenient.

SUMMARY

The principles of ultraviolet absorption spectrophotometry are discussed with reference to the application of spectrographic methods to the quantitative analysis of carcinogenic hydrocarbons and their metabolic products in tissue extracts. The data available concerning the absorption spectra of carcinogenic hydrocarbons and related substances are reviewed and the factors limiting the accuracy and sensitivity of the method are considered. In tissue extracts the presence of background absorption due to other absorbing constituents is a major factor in limiting the value of spectrographic analysis of such extracts and a rapid method of estimating approximately the degree to which such background interference is present in any given case is presented.

The author wishes to express his appreciation of the interest and encouragement of Professor L. F. Fieser and to thank Mr. John Clarke and Mrs. J. V. Burkhead for technical assistance.

REFERENCES

1. Boyland, E., and A. A. Levi. Metabolism of Polycyclic Compounds. I. Production of Dihydroxydihydroanthracene from Anthracene. Biochem. J., **29**:2679-2683. 1935.
2. Boyland, E., A. A. Levi, E. H. Mawson, and E. Roe. Metabolism of Polycyclic Compounds. 4. Production of Dihydroxy-1:2:5:6-dibenzanthracene from 1:2:5:6-Dibenzanthracene. Biochem. J., **35**:184-191. 1941.
3. Capper, N. S., and J. K. Marsh. Absorption Spectra of Condensed Nuclear Hydrocarbons. J. Chem. Soc., 724-730. 1926.
4. Cason, J., and L. F. Fieser. Synthesis of 4′,8′-Dihydroxy-1,2,5,6-dibenzanthracene and its Relation to Products of Metabolism of the Hydrocarbon. J. Am. Chem. Soc., **62**: 2681-2687. 1940.
5. Chalmers, J. G. The Role of 1:2:5:6-Dibenzanthracene in the Production of Fowl Tumours. Biochem. J., **28**: 1214-1218. 1934.
6. Chalmers, J. G., and H. M. Kirby. The Elimination of 3,4-Benzpyrene from the Animal Body after Subcutaneous Injection. I. Unchanged Benzpyrene. Biochem. J., **34**:1191-1195. 1940.

[4] In the presence of continuous background absorption the resolution of the fine structure absorption bands is decreased and the maxima may be suppressed. This effect will counteract the increase in intensity due to the additive effect of the background. Other factors may also be present in some extracts which affect the intensity. If the solutions are not optically clear there will be a loss of light from scattering by suspended particles which will enhance the apparent absorption. This effect will be very much greater at shorter wave lengths since, according to the Rayleigh equation, the intensity of the scattered light is a linear function of the inverse fourth power of the wave length.

7. Clar, E. Zur Kenntnis mehrkerniger aromatischer Kohlenwasserstoffe und ihrer Abkömmlinge, I. Mitt.: Dibenzanthracene und ihre Chinone. Ber. deutsch. chem. Ges., **62**:350-359. 1929.
8. Dobriner, K., G. Lavin, and C. P. Rhoads. Conversion of 1,2,5,6-Dibenzanthracene by Rabbits, Rats and Mice. Significance in Carcinogenesis of this Conversion. Proc. Soc. Exper. Biol. & Med., **41**:67-69. 1939.
9. Dobriner, K., G. Lavin, and C. P. Rhoads. The Spectroscopic Study of Biological Extracts. I. Urine. Cancer Research, **2**:79-94. 1942.
10. Dobriner, K., C. P. Rhoads, and G. I. Lavin. The Spectroscopic Study of Biological Extracts. II. The Detection, Isolation, and Biological Effects of the Metabolites of 1,2,5,6-Dibenzanthracene. Cancer Research, **2**:93-107. 1942.
11. Fieser, L. F., and R. C. Clapp. Synthesis of 10-Methyl-3′-isopropyl-1,2-benzanthracene from 9,10-Dihydroretene. J. Am. Chem. Soc., **63**:319-323. 1941.
12. Fieser, L. F., and E. B. Hershberg. Reduction and Hydrogenation of Methylcholanthrene. J. Am. Chem. Soc., **60**: 940-946. 1938.
13. Fieser, L. F., and E. B. Hershberg. Further Observations on the Use of Hydrogen Fluoride in Acylations and Cyclizations. J. Am. Chem. Soc., **62**:49-53. 1940.
14. Fieser, L. F., and W. S. Johnson. Syntheses in the 1,2-Benzanthracene and Chrysene Series. J. Am. Chem. Soc., **61**:1647-1654. 1939.
15. Holiday, E. R. Spectrophotometry of Proteins. I. Absorption Spectra of Tyrosine, Tryptophan and their Mixtures. Biochem. J., **30**:1795-1799. 1936.
16. Jones, R. N. The Ultraviolet Absorption Spectra of Some Derivatives of 1,2-Benzanthracene. J. Am. Chem. Soc., **62**:148-152. 1940.
17. Jones, R. N. Further Observations on the Absorption Spectra of Derivatives of 1,2-Benzanthracene. J. Am. Chem. Soc., **63**:151-155. 1941.
18. Jones, R. N. Note on the Absorption Spectra of Some Alkyl Chrysenes. J. Am. Chem. Soc., **63**:313-314. 1941.
19. Jones, R. N. The Spectrographic Analysis of Carcinogenic Hydrocarbons and Metabolites. II. Determination of 1,2,5,6-Dibenzanthracene and 4′,8′-Dihydroxy-1,2,5,6-Dibenzanthracene in Rat Excreta. Cancer Research, **2**: 245-251. 1942.
20. Jones, R. N. The Spectrographic Analysis of Carcinogenic Hydrocarbons and Metabolites. III. Distribution of 1,2,5,6-Dibenzanthracene in Rats following Subcutaneous Injection in Olive Oil. Cancer Research, **2**:252-255. 1942.
21. Lorenz, E., and M. J. Shear. Studies in Carcinogenesis. II. The Detection of Dibenzanthracene in Mouse Tumors Induced by this Hydrocarbon. Am. J. Cancer, **26**:333-343. 1936.
22. Mayneord, W. V., and E. M. F. Roe. The Ultraviolet Absorption Spectra of Some Complex Aromatic Hydrocarbons. I. Proc. Roy. Soc., London, s. A., **152**:299-324. 1935.
23. Mayneord, W. V., and E. M. F. Roe. The Ultraviolet Absorption Spectra of Some Complex Aromatic Hydrocarbons. II. Proc. Roy. Soc., London, s. A., **158**:634-650. 1937.
24. Mohler, H., and J. Sorge. Ultraviolettabsorption einiger polycyclischer Kohlenwasserstoffe. Helv. Chim. Acta, **22**:229-235. 1939.
25. Shear, M. J. Studies in Carcinogenesis. I. The Production of Tumors in Mice with Hydrocarbons. Am. J. Cancer, **26**:322-332. 1936.
26. Twyman, F., and C. B. Allsopp. The Practice of Spectrophotometry (140 pages), Adam Hilger, Ltd., London, 1934.
27. Wilkie, J. B. Physical and Chemical Determination of Vitamin A. Indust. & Engineering Chem. (Analytical Edition), **13**:209-211. 1941.

The Spectrographic Analysis of Carcinogenic Hydrocarbons and Metabolites

II. Determination of 1,2,5,6-Dibenzanthracene and 4',8'-Dihydroxy-1,2,5,6-Dibenzanthracene in Rat Excreta*

R. Norman Jones, Ph.D.

(From the Converse Memorial Laboratory, Harvard University, Cambridge, Mass.)

(Received for publication January 7, 1942)

Dobriner, Rhoads, and Lavin (8, 9) isolated from the excreta of rats injected subcutaneously with 1,2,5,6-dibenzanthracene [1] a phenolic compound which was later shown by Cason and Fieser (3) to have an absorption spectrum identical with that of synthetic 4',8'-dihydroxy-1,2,5,6-dibenzanthracene (Fig. 1); the same phenolic derivative was also present in the excreta of mice which had received similar injections. From the excreta of rabbits injected with the same hydrocarbon an isomeric dihydroxy-1,2,5,6-dibenzanthracene was obtained which had a different spectrum. Earlier, Boyland and co-workers (1, 2) had investi-

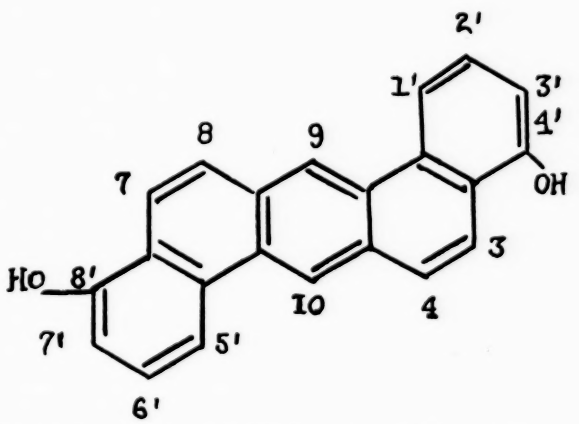


FIG. 1.—Formula I, 4',8'-dihydroxy-1,2,5,6-dibenzanthracene.

gated the metabolism of anthracene and of dibenzanthracene following oral administration to rats and to rabbits and had isolated the same dihydroxy-1,2,5,6-dibenzanthracene from rabbit urine. Boyland, Mawson, Levi, and Roe (2) suggested that the rabbit metabolite might be 4',8'-dihydroxy-1,2,5,6-dibenzanthracene on account of certain features of the spectrum of the corresponding 9,10-quinone, although the possibility that the hydroxyl groups are at the 3- and 7- positions or the 3- and 8- positions was not excluded.

Meanwhile Cason and Fieser (4) synthesized 3,7-dihydroxy-1,2,5,6-dibenzanthraquinone which differs from the quinone obtained by oxidation of the rabbit metabolite, the structure of which is therefore still uncertain.

The yields of phenolic metabolite obtained from rabbits following subcutaneous injection are considerably greater than those obtained from rats and mice (9) and such species differences are of interest in view of the fact that while rats and mice are susceptible to the carcinogenic action of dibenzanthracene, rabbits are much more resistive and continued subcutaneous injection of the hydrocarbon causes acute liver necrosis or chronic liver degeneration and cirrhosis, but no suggestion of tumor formation near the site of injection (7). Quantitative studies of the relative efficiency of the detoxification process of different species and of the same species under varying dietary conditions merit investigation to see if there is any correlation between the rapidity and completeness of detoxification and the species difference, genetic strain, or other factors known to influence the onset of hydrocarbon carcinogenesis. Such an approach receives encouragement from the observations of Dobriner, Rhoads, and Lavin (9) and of Boyland, Levi, Mawson, and Roe (2) that the phenolic metabolite obtained from rabbits is not carcinogenic to mice. Fieser had noted (3, 7) that the introduction of a hydroxyl group into a carcinogenic hydrocarbon usually destroys the carcinogenic activity or at least reduces it considerably. Thus the process of conversion of dibenzanthracene into a phenolic metabolite is a true detoxification and not merely an elimination process.

EXPERIMENTAL OBSERVATIONS

A. Feces

The experiments described in this and the succeeding paper are of a preliminary nature and were undertaken to investigate whether it is practical to determine dibenzanthracene and 4',8'-dihydroxy-1,2,5,6-

* This investigation was aided by a grant from The International Cancer Research Foundation.

[1] Subsequently referred to as dibenzanthracene.

dibenzanthracene spectrographically in the excreta and tissue extracts of rats which have received subcutaneous injections of the hydrocarbon in olive oil. Through the kindness of Dr. Rhoads and Dr. Dobriner of the Memorial Hospital, New York, we obtained 13 liters of urine and 1,800 gm. of feces collected from 32 rats which had received subcutaneous injections of dibenzanthracene in olive oil over a period of several weeks. The total amount of hydrocarbon which had been injected was 4.5 gm. and the excreta had been collected with the object of providing material for the isolation of the phenolic metabolite.

Feces.—Twenty grams of the feces, obtained as 6 separate samples from various parts of the bulked material, were ground with anhydrous sodium sulfate (10 gm.) and extracted for 72 hours with peroxide-free ether in a Soxhlet extractor. At the end of this time the fresh extracts were no longer fluorescent, and it was assumed that extraction was complete. The bulked ethereal extract (500 ml.), which possessed a strong blue fluorescence, was extracted 5 times with a solution containing 5 per cent sodium bicarbonate and 5 per cent sodium carbonate to yield alkaline extract A. The ethereal phase was next extracted 5 times with 50 ml. of 10 per cent sodium carbonate solution, giving alkaline extract B, and the residual ether was extracted 5 times with 50 ml. of N sodium hydroxide solution yielding a phenolic extract, and a residual ether extract containing the neutral fraction.

The phenolic extract.—This possessed a pronounced yellowish fluorescence in ultraviolet light and was saturated with carbon dioxide and extracted with ether. The yellow fluorescence of the aqueous phase disappeared during this process and the ether extracts acquired a blue fluorescence. Saturation with carbon dioxide was continued until no further fluorescent material was extracted by ether. The ethereal layer was washed several times with water, dried with anhydrous sodium sulfate, and on removal of the solvent in a stream of nitrogen a semisolid colorless fluorescent oil (45 mgm.) remained, possessing a strong phenolic odor (the phenolic fraction).

The alkaline extracts A and B.—These extracts were nonfluorescent and were neutralized with 6N hydrochloric acid and extracted with ether. Removal of the solvent from the washed and dried ethereal layer yielded nonfluorescent residues which were not further investigated. The alkaline extract B, which contained the bulk of the fatty acids, emulsified badly during this process.

The neutral fraction.—This fraction, which retained most of the fluorescence of the original extract, was washed with water, and dried with anhydrous sodium sulfate. On removal of the solvent in a stream of nitrogen, there remained 333 mgm. of a brown oil. In a second experiment on a similar sample using a slightly different extraction procedure 248 mgm. of neutral fraction and 75 mgm. of phenolic fraction were obtained.

The absorption spectrum of the neutral fraction was determined in ethanol solution, the intensity being calculated as $E^{1\%}_{1\ cm.}$ based on the weight of the extract. The curve (Fig. 2-*A*) shows a fair resemblance to that of pure dibenzanthracene (Fig. 2-*B*) which is fitted to the observed curve in the manner described in the previous paper (11). The concentration of dibenzanthracene in the extract, calculated from the intensity of the maximum at 2,970 A. may be accepted as reasonably accurate (Table I).

TABLE I: ANALYSIS OF NEUTRAL FRACTIONS OF FECES AND URINE

	Weight of fraction, in mgm.	$E^{1\%}_{1\ cm.}$ 2,970 A.	Dibenzanthracene found	
			Per cent	Mgm.
Feces (20 gm.)				
Experiment 1	248	63	1.0	2.5
After acid hydrolysis	210	72	1.14	2.4
After alkaline saponification	160	90	1.43	2.3
Experiment 2	333	43.5	0.7	2.3
Urine (200 ml.)				
	90	22	0.35	0.31

The neutral fraction of urinary ethereal extracts from rabbits which had received oral administration of anthracene were found by Boyland and Levi (2) to contain a dihydroxydihydro derivative of anthracene which was decomposed by warm aqueous hydrochloric acid to yield 1-anthrol. Although the spectrum of the neutral fraction obtained above did not suggest the presence of a similar derivative of dibenzanthracene, this was investigated further. The neutral fraction (248 mgm.) was refluxed for one hour with N hydrochloric acid in 50 per cent aqueous ethanol and, after cooling and diluting with water, the product was extracted with ether. Almost all of the fluorescent material passed into the ethereal phase, which was washed with N sodium hydroxide solution and water and dried with anhydrous sodium sulfate. On removal of the solvent in a stream of nitrogen there remained 210

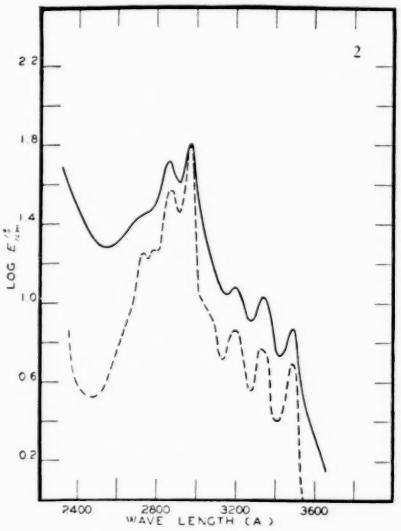


FIG. 2.—*A*, continuous line ———, neutral fraction from rat feces. *B*, dash line – – – –, master curve of 1,2,5,6-dibenzanthracene.

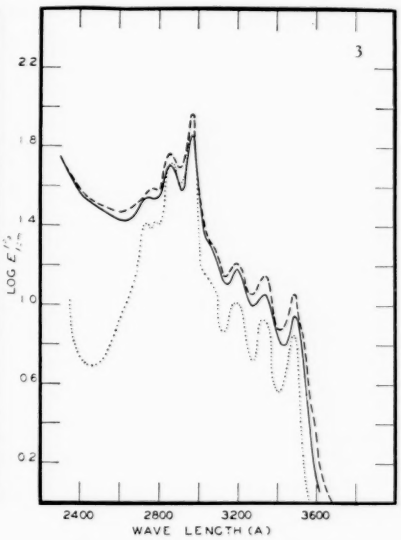


FIG. 3.—*A*, continuous line ———, rat feces, neutral fraction after acid hydrolysis. *B*, dash line – – – –, same as *A* after alkaline saponification. *C*, dotted line . . . . ., master curve of 1,2,5,6-dibenzanthracene fitted to curve *B*.

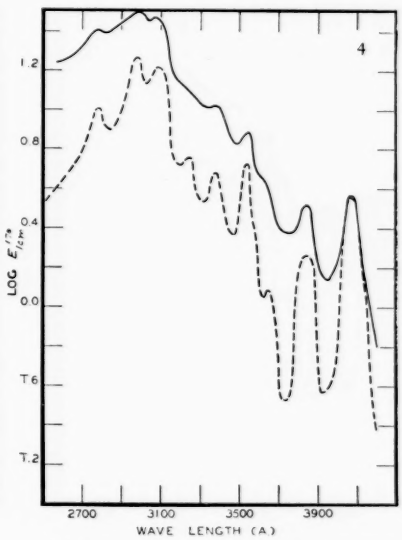


FIG. 4.—*A*, continuous line ———, phenolic fraction from rat feces. *B*, dash line – – – –, master curve of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene.

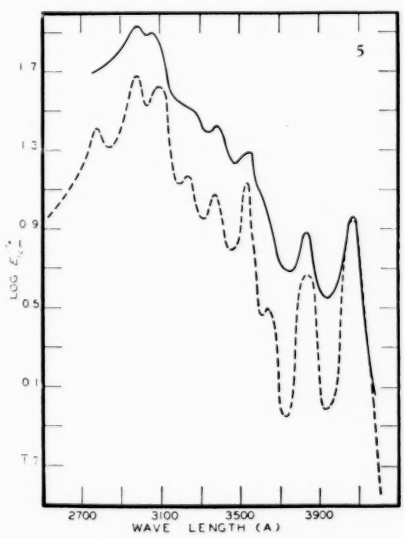


FIG. 5.—*A*, continuous line ———, high vacuum distillation of phenolic fraction from rat feces, fraction II. *B*, dash line – – – –, master curve of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene.

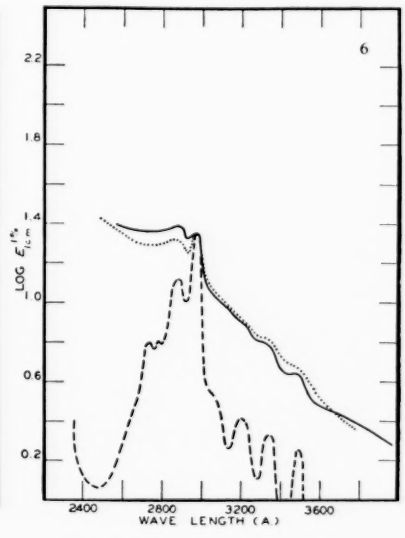


FIG. 6.—*A*, continuous line ———, neutral fraction from rat urine. *B*, dash line – – – –, master curve of 1,2,5,6-dibenzanthracene. *C*, dotted line . . . . ., neutral fraction after acid hydrolysis.

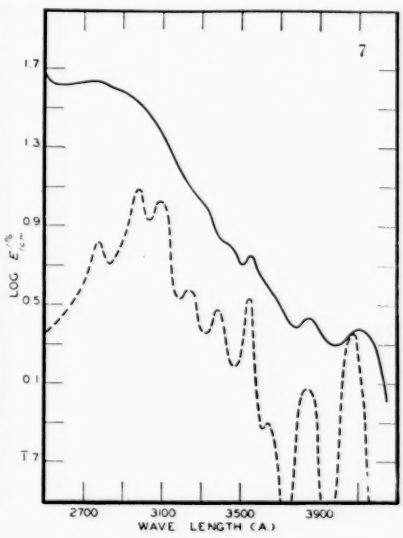


FIG. 7.—*A*, continuous line ———, phenolic fraction from rat urine. *B*, dash line – – – –, master curve of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene.

mgm. of a fluorescent gum, the spectrum of which is given in Fig. 3-*A*. The somewhat greater intensity of the absorption maximum of this extract compared with that of Fig. 2-*A* just counterbalances the loss of weight and indicates that treatment with acid has removed no chromophoric material.

The extract remaining from the acid treatment was next dissolved in 50 ml. of ethanol and saponified for 2 hours with 40 ml. of N potassium hydroxide in 90 per cent ethanol. At the conclusion of the reaction the cooled solution was diluted with water extracted with ether and the ethereal extract washed first with N hydrochloric acid solution, then with water, and dried. Removal of the solvent left 160 mgm. of a fluorescent gum the spectrographic data on which are given in Fig. 3-*B* and Table I. The saponification process removed 50 mgm. of nonchromophoric material but influenced neither the shape of the curve nor the total amount of substances responsible for the absorption spectrum. It may therefore be concluded that the characteristic absorption of the neutral fraction is due entirely to hydrocarbon, together with some background absorption, neither of which is greatly influenced by acid hydrolysis or alkaline saponification. Any other neutral metabolites which are sensitive to acid or alkali can only be present in traces which are too small to influence the spectrum.[2]

The phenolic fraction.—The absorption spectrum of the phenolic fraction in ethanol is given in Fig. 4-*A*. Comparison with the curve of dibenzanthracene shows several differences, particularly in the region near 3,000 A. and in the presence of a characteristic maximum at 4,070 A. which is beyond the absorption threshold of dibenzanthracene on the long wave length side. These differences were observed by Dobriner, Rhoads, and Lavin (9) by their qualitative technic and led them to the isolation of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene, the master curve of which (Fig. 4-*B*) is fitted to the curve in Fig. 4-*A*. The coincidence of the curves in Fig. 4-*A* and 4-*B* is not as close as that found between Fig. 2-*A* and 2-*B* which suggests that the phenolic fraction contains more interfering chromophoric substances.

The bulked phenolic fractions from two extractions (120 mgm.) were submitted to high vacuum distillation yielding 3 fractions, the data for which are given in Table III and Fig. 5-*A*. The first fraction (75 mgm.) which distilled at low temperature formed a semicrystalline colorless mass with a strong phenolic odor, consisting chiefly of phenol and cresols, and was not examined further. Fraction II (19 mgm.) was highly fluorescent and gave a well defined spectrum of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene. The content of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene in the extract before distillation was 710γ in 120 mgm., while the amount calculated for the second fraction of the distillate was 380γ in 19 mgm. Assuming the quantitative analyses are equally accurate this represents an increase in concentration from 0.6 to 2.0 per cent. The total amount of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene recovered from fraction II was only 53 per cent of that calculated as present in the original extract. The discrepancy may be due in part to an error in the estimation of the concentration in the original extract due to enhancement of the intensity by the phenol and cresol contribution, but such com-

Table II: Analysis of Phenolic Fractions of Feces and Urine

	Weight of fraction, in mgm.	$E^{1\%}_{1\text{ cm.}}$ 4,070 A.	4′,8′-Dihydroxy-1,2,5,6-dibenzanthracene found	
			Per cent	Mgm.
Feces (20 gm.)				
Experiment 1	75	2.1	0.45	0.34
Experiment 2	45	3.8	0.83	0.37
Urine (200 ml.)				
	33	2.4	0.52	0.17

Table III: High Vacuum Distillation of Phenolic Fraction from Feces

Weight of material before distillation 120 mgm.

Fraction	Weight in mgm.	Temperature	$E^{1\%}_{1\text{ cm.}}$ 4,070 A.	4′,8′-Dihydroxy-1,2,5,6-dibenzanthracene found, per cent	Weight in mgm.
I	75	20-100°	..	..	...
II	19	110-250°	9.1	2.0	0.38
III	12	residue	..	..	...

[2] This conclusion is valid only for neutral metabolites which can be extracted from the feces with ether. Glycuronic acid and mercapturic acid derivatives, if present, would not be expected to dissolve in ether and would remain in the nonextractible residue of the feces. This fraction has not been investigated; however, one would expect to find such derivatives in urinary rather than fecal extracts.

pounds are unlikely to influence the absorption at 4,070 A. to any considerable extent since they are colorless and only absorb much further in the ultraviolet. On the other hand it is possible that considerable destruction of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene may have occurred during the distillation process. In subsequent calculations discussed later in the paper the concentrations determined before high vacuum distillation are accepted with the reservation that they may err on the high side.

B. Urine

A sample of the strongly fluorescent urine (200 ml.) was rendered faintly acid with 2N hydrochloric acid and extracted with ether in a continuous extractor for 96 hours, by which time the fresh extracts were no longer fluorescent. The ethereal solution was separated into three alkaline fractions (A, B, and phenolic) and a neutral fraction by a procedure which differed slightly from that employed for the extraction of the feces.[3]

The alkaline extracts A and B showed no pronounced fluorescence and were not further examined. The phenolic extract (25 mgm.) was colorless and had a strong blue fluorescence while the neutral fraction 90 mgm. had a greenish blue fluorescence.

The spectrum of the neutral fraction (Fig. 6-*A*) shows certain characteristic features of the dibenzanthracene spectrum (Fig. 6-*B*) but is evidently modified by much background absorption. The concentration of dibenzanthracene calculated from the intensity of the maximum at 2,970 A. is probably considerably in excess of the amount actually present.

A part of the neutral fraction (49 mgm.) was refluxed with N hydrochloric acid in 50 per cent ethanol under conditions identical with those used for the corresponding fecal extract, yielding a neutral fraction (39 mgm.) (Fig. 6-*C*) the spectrum of which differed little from that obtained before acid treatment, indicating that acid hydrolysis did not appreciably affect either the substance responsible for the characteristic spectrum or those responsible for the background.

The spectrum of the phenolic extract (Fig. 7-*A*) shows distinct evidence of the presence of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene but is influenced considerably by background absorption. On the basis of the intensity of the maximum at 4,070 A. the amount of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene present is only 170γ. This extract was not processed further.

Urine residue not soluble in ether.—To investigate whether the urine contained glycuronic acid derivatives of dibenzanthracene the residue from the neutral ether extraction (which had a faintly alkaline reaction) was acidified with 20 ml. of 6N hydrochloric acid and allowed to stand for 24 hours at room temperature and then reextracted with ether in a continuous extractor for 48 hours. The ethereal extract had a pale yellow color and very faint fluorescence. It was worked up into neutral and phenolic fractions neither of which showed any appreciable fluorescence nor gave any reaction for glycuronic acids with the naphtharesorcinol reagent. The remaining nonextractable urine residue also failed to give a reaction with the naphtharesorcinol reagent nor did the original urine. The failure to detect any conjugated metabolites following injection of dibenzanthracene into rats parallels the observations of Dobriner, Rhoads, and Lavin (9). Recently Boyland and co-workers (3) have demonstrated an increase in the excretion of ethereal sulfates in the urine following the feeding of dibenzanthracene to rats and to rabbits, but this indirect evidence favoring a mercapturic acid structure for a hypothetical precursor of the phenolic metabolite has not yet been substantiated by more direct evidence.

DISCUSSION

Assuming that the samples of feces and urine examined were truly representative of the whole, the total amount of hydrocarbon and metabolite in the excreta may be calculated from the spectrographic data. This has been done in Table IV. The urine sample was obtained from the well mixed liquid and is therefore unlikely to be subject to sampling errors, while the close agreement of two independent determinations on the feces are suggestive of satisfactory sampling and of the reproducibility of the results. The comparison of the shape of the absorption curves by the method described in the previous paper (11) indicates that the analyses of the neutral extracts of the feces are probably fairly accurate while those of the other extracts may err on the high side.

The experiments described are to be regarded primarily as a demonstration of the analytical methods. The injection experiments were planned with the object of providing large amounts of excretory material for qualitative work rather than for precise quantitative investigations and, provided these reservations are born in mind, the results summarized in Table IV illustrate several significant points.

[3] After removing the alkaline extract A with 5 per cent sodium carbonate-5 per cent sodium bicarbonate buffer, the ethereal phase was extracted with N sodium hydroxide solution and the extract, after neutralization with N hydrochloric acid, reextracted with ether. Removal of the solvent, after first washing and drying, yielded 195 mgm. of a waxy solid which was redissolved in ether and extracted with 10 per cent sodium carbonate solution yielding an aqueous phase containing alkaline extract B as sodium salts and leaving the phenolic fraction in the ethereal layer.

Of the total of 4.5 gm. of hydrocarbon administered to the rats, only 43 mgm. are detectable as a phenolic metabolite and 231 mgm. as unchanged dibenzanthracene. Of this excreted material by far the greater proportion is found in fecal extracts and relatively little in the urine. Dobriner, Rhoads, and Lavin (9) report qualitative observations in substantial agreement with the above as to the small amount of hydrocarbon and metabolite which can be detected in the excreta and the relative distribution between the feces and the urine. Chalmers and Kirby (6) have observed that unchanged 3,4-benzpyrene is excreted to some extent in the feces following subcutaneous injection, there being little in the urine. Chalmers (5) has also reported that both in the urine and feces of rats injected intravenously with colloidal suspensions of 3,4-benzpyrene alkali-soluble fluorescent derivatives are present, and, as Peacock suggested (13), the hydrocarbon and the phenolic derivative are probably excreted into the intestinal tract with the bile. Chalmers has emphasized the differences between the results obtained from feeding experiments and from injection experiments (5) and his comments have since received further significance from the work of Lorenz and Stewart (12) who have investigated the fate of dibenzanthracene fed to rats, and who were unable to detect any fluorescent material in the gastrointestinal tract below the beginning of the large intestine. As yet, however, no strictly comparative study of feeding and injection experiments has been carried out in the same laboratory using the same analytical technic.

TABLE IV: TOTAL AMOUNT OF HYDROCARBON AND METABOLITE RECOVERED FROM FECES AND URINE

1,2,5,6-Dibenzanthracene in 1800 gm. feces and 13 liters of urine calculated from data in Table I.

Feces	Urine	Total	Total injected
211 mgm.	20 mgm.	231 mgm.	4.5 gm.

4′,8′-Dihydroxy-1,2,5,6-dibenzanthracene in 1,800 gm. feces and 13 liters of urine calculated from data in Table II.

Feces	Urine	Total
32 mgm.	11 mgm.	43 mgm.

The very low efficiency of the excretory process indicated by the results in Table IV may be due to several causes. One contributing factor, no doubt, is the encapsulation of injected material in vesicles near the site of injection. This is discussed more fully in the succeeding paper and it would appear from qualitative tests that suitable modification of the injection technic may greatly reduce this. It has been assumed during the extraction procedures employed so far that the metabolites of the hydrocarbon will retain some fluorescence and spectrographic examination has been restricted to those extracts which exhibited strong fluorescence. While this is a useful expedient it may be misleading and the greater part of the metabolized hydrocarbon may be present in a nonfluorescing fraction. The influence of substituents on the fluorescence of polynuclear aromatic hydrocarbons has not been systematically investigated but it may be predicted that the introduction of several types of groups may completely destroy the fluorescence. As examples of this effect it may be suggested that reduction of dibenzanthracene at the 9- and 10- positions would yield dihydro derivatives, the fluorescence of which would be greatly reduced. The opening of any of the rings by oxidative fission would probably destroy the fluorescence completely, except perhaps in the case of the terminal rings.

The dosage is also a factor which may influence the efficiency of the excretory process. Obviously the conditions operating in the experiments discussed here are highly unphysiological and small amounts of carcinogenic substances such as may be imagined to occur in the normal organism, perhaps as side products of sterol metabolism, may be removed rapidly and completely by a mechanism which fails to cope with large excesses of carcinogens introduced artificially. This may become evident if, with the increasing development of microanalytical technic it becomes possible to reduce the dosage of injected material to an amount with which the organism can deal. Under such conditions an increase in the efficiency of the detoxification mechanism may manifest itself as an increase in the ratio of excreted metabolite to unchanged hydrocarbon.

The greater part of the unchanged hydrocarbon may also be oxidized to small fragments which may be excreted in forms not easily recognized as metabolites of the original hydrocarbon unless marked by an isotopic or radioactive atom.

SUMMARY

Spectrographic analyses of the feces and urine of rats which have received injections of 1,2,5,6-dibenzanthracene in olive oil indicate that, of 4.5 gm. of hydrocarbon injected into 32 rats over a period of several weeks, only 231 mgm. could be detected in the excreta in an unchanged form and 43 mgm. as 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene. Of this excreted material the greater part was found in the feces. The shape of the absorption curves suggests that the quantitative data on the hydrocarbon in the feces is more accurate than the other data, which are likely to err on the high side.

The author wishes to express his appreciation of the interest and encouragement of Professor L. F. Fieser during the course of this work.

REFERENCES

1. Boyland, E., and A. A. Levi. Metabolism of Polycyclic Compounds. I. Production of Dihydroxydihydroanthracene from Anthracene. Biochem. J., **29**:2679-2683. 1935.
2. Boyland, E., A. A. Levi, E. H. Mawson, and E. Roe. Metabolism of Polycyclic Compounds. 4. Production of Dihydroxy-1:2:5:6-dibenzanthracene from 1:2:5:6-Dibenzanthracene. Biochem. J., **35**:184-191. 1941.
3. Cason, J., and L. F. Fieser. Synthesis of 4′,-8′-Dihydroxy-1,2,5,6-dibenzanthracene and its Relation to Products of Metabolism of the Hydrocarbon. J. Am. Chem. Soc., **62**: 2681-2687. 1940.
4. Cason, J., and L. F. Fieser. 3,7-Dihydroxy-1,2,5,6-dibenzanthraquinone. J. Am. Chem. Soc., **63**:1256-1258. 1941.
5. Chalmers, J. G. The Elimination of 3,4-Benzpyrene from the Rat. Biochem. J., **34**:678-684. 1940.
6. Chalmers, J. G., and H. M. Kirby. The Elimination of 3,4-Benzpyrene from the Animal Body after Subcutaneous Injection. I. Unchanged Benzpyrene. Biochem. J., **34**: 1191-1195. 1940.
7. Claude, A. Liver Degeneration and Cirrhosis Produced by 1,2,5,6-Dibenzanthracene. Am. J. Cancer, **31**:100-103. 1937.
8. Dobriner, K., G. Lavin, and C. P. Rhoads. Conversion of 1,2,5,6-Dibenzanthracene by Rabbits, Rats and Mice. Significance in Carcinogenesis of this Conversion. Proc. Soc. Exper. Biol. & Med., **41**:67-69. 1939.
9. Dobriner, K., C. P. Rhoads, and G. I. Lavin. The Spectroscopic Study of Biological Extracts. II. The Detection, Isolation, and Biological Effects of the Metabolites of 1,2,5,6-Dibenzanthracene. Cancer Research, **2**:95-107. 1942.
10. Fieser, L. F. Carcinogenic Activity, Structure and Chemical Reactivity of Polynuclear Hydrocarbons. Am. J. Cancer, **34**:37-124. 1938.
11. Jones, R. N. The Spectrographic Analysis of Carcinogenic Hydrocarbons and Metabolites. I. Introduction. Cancer Research, **2**:237-244. 1942.
12. Lorenz, E., and H. L. Stewart. Intestinal Carcinoma and Other Lesions in Mice Following Oral Administration of 1,2,5,6-Dibenzanthracene and 20-Methylcholanthrene. J. Nat. Cancer Inst., **1**:17-40. 1940.
13. Peacock, P. R. Evidence Regarding the Mechanism of Elimination of 1,2-Benzpyrene, 1,2,5,6-Dibenzanthracene and Anthracene from the Blood Stream of Injected Animals. Brit. J. Exper. Path., **17**:164-172. 1936.

The Spectrographic Analysis of Carcinogenic Hydrocarbons and Metabolites

III. Distribution of 1,2,5,6-Dibenzanthracene in Rats Following Subcutaneous Injection in Olive Oil*

R. Norman Jones, Ph.D.

(From the Converse Memorial Laboratory, Harvard University, Cambridge, Mass.)

(Received for publication January 7, 1942)

The experiments described in this paper were undertaken with the object of investigating the distribution of 1,2,5,6-dibenzanthracene in the rat following subcutaneous injection of the hydrocarbon in olive oil. Analyses of the excreta of rats which had received similar injections were discussed in the previous paper (4), where it was observed that only a very small percentage of the total hydrocarbon injected could be detected, either as unchanged hydrocarbon or as a phenolic metabolite, 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene. The results described here show that an appreciable amount of the injected hydrocarbon may remain encapsulated near the site of injection for a considerable time but there is also qualitative evidence that such encapsulation may be greatly reduced by modification of the injection technic.

The analyses were carried out on two rats[1] each of which had received 8 injections of 20 mgm. of dibenzanthracene in olive oil at weekly intervals during the 8 weeks immediately preceding the autopsy.

MATERIALS AND METHODS

Post-Mortem Examination of the Rats

Rat No. 1 (female) had multilocular abscesses on both flanks which showed on fluorescence in ultraviolet light. On opening the body cavity a perirenal cystic structure (2 cm. diam.) was present which contained a clear fluid which fluoresced in ultraviolet light as also did the wall of the sac. This cyst, which presumably was caused by the accidental penetration of the perirenal tissue during injection, was excised, and on opening it an oily fluid exuded. The contents were collected in ether and stored together with the sac itself.

The viscera were grossly normal as were the following organs: heart, lung, thymus, salivary gland, thyroid, liver, spleen, pancreas, kidney, colon, bladder, ovary, adrenal, stomach, brain, cerebellum, pituitary, bone, bone marrow, all of which were examined grossly by ultraviolet light without observing any pronounced fluorescence in any of the organs such as might indicate a local concentration of carcinogenic hydrocarbon. The intestines and urinary bladder, however, showed a distinct blue fluorescence. The subcutaneous tissue of the back and sides was riddled with fluorescent vesicles containing oily material.

Rat No. 2 (male) resembled the first rat closely except for the absence of a perirenal sac. The testicles of rat No. 2 showed no gross fluorescence. The hair of both rats fluoresced brilliantly but this was not due to the effect of the hydrocarbon since the hair of control rats had a similar fluorescence.

The skins were removed from both rats, the carcasses being carefully washed with ether so as to collect quantitatively the oily fluid which exuded from the vesicles. The dissecting trays were also washed with ether, the washings being added to those obtained above. The greater part of the hair was removed from the skin and collected separately, after which the skins were added to the ether washings from the body cavity and stored.

After removal of the livers which were stored separately, the carcasses were preserved under ether together with the various organs which had been examined for fluorescence. The tissues were divided as follows into five separate samples for subsequent extraction and spectrographic analysis: A. hair; B. livers; C. skins together with ether washings of the body cavity; D. carcasses and remaining organs; E. perirenal sac and part of the kidney of rat No. 1. These samples were stored under ether at 5° C. until required for spectrographic analysis.

* This investigation was aided by a grant from The International Cancer Research Foundation.

[1] Kindly supplied by Dr. K. Dobriner of the Memorial Hospital, New York.

Extraction and Spectrographic Analysis of Organs

With the exception of the hair, the various fractions were prepared for spectrographic analysis in the following manner. The ether washings were decanted and preserved, the residual tissue washed twice with ether, this ether being added to that collected above, and the tissue minced with an equal weight of anhydrous sodium sulfate and extracted with chloroform until the fresh chloroform extracts were no longer fluorescent. The extraction of samples B and E was carried out in a Soxhlet extractor while samples C and D were extracted in a modified Wolfe-Hershberg extractor (2) adapted for the extraction of solid material. Fraction A was extracted with chloroform in a Soxhlet extractor without the addition of sodium sulfate.

The chloroform extracts so obtained from the several fractions were added to the respective ethereal washings and the solvent removed in a stream of nitrogen under reduced pressure. The residues were taken up in ether and extracted three times with N sodium hydroxide solution. The alkaline solution was back extracted 4 times with ether, the ether extracts bulked, washed with water, dried with anhydrous sodium sulfate, and the solvent removed to leave the neutral fraction.

The aqueous alkaline extracts were saturated for 2 to 3 hours with a rapid stream of carbon dioxide and reextracted with ether. The ether extract so obtained was reextracted with alkali and the second alkaline extract reacidified with carbon dioxide and reextracted with ether. After washing with water, drying with anhydrous sodium sulfate, and removing the solvent in a stream of nitrogen under reduced pressure there remained the phenolic fraction. The double precipitation with carbon dioxide described here was necessary since the first treatment with N sodium hydroxide caused some emulsification and two treatments were found necessary to free the phenolic fraction from unchanged dibenzanthracene.

The results of the spectrographic analyses are summarized in Table I from which it will be seen that a considerable quantity of unchanged hydrocarbon (36 per cent) is recoverable from the bodies and organs of the rats. Comparison of the curves for the various neutral fractions with the master curve for the pure hydrocarbon by the method described in an earlier publication (3) (Figs. 1 to 5) indicates that the distortion of the curve due to background absorption is very small in the cases of the extracts from the perirenal sac (E), the skin (C), and the neutral extract of the bodies and other organs (D). The liver extract (B) shows very pronounced distortion, and the concentration of dibenzanthracene in the livers as calculated from this curve (Fig. 2) is probably much too high.

The phenolic fractions after removal of the solvent were distilled in a current of steam until 500 ml. of aqueous distillate had collected, in order to remove volatile phenolic substances. The spectra of the residual nonvolatile fractions showed no trace of any structure which could be attributed to 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene. The curves from all fractions, however, had a similar shape with a broad indeterminate maximum between 2,700 and 2,800 A.; an example of the spectrum of one of these extracts is given in Fig. 6. All the phenolic fractions showed a distinct greenish blue fluorescence but unlike the blue fluorescence characteristic of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene this fluorescence did not change to a yellowish color on addition of aqueous alkali. Experience of other work of a similar nature has convinced the author that the presence of blue fluorescence in an extract, unless accompanied by a characteristic absorption spectrum, cannot be accepted as indicative of the presence of carcinogenic hydrocarbons or their metabolites in fat-soluble tissue extracts of this kind. It appears therefore that in none of these body extracts was any trace of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene detected.

The observations reported here are in agreement

Table I: Spectrographic Analysis of 1,2,5,6-Dibenzanthracene in Tissue Extracts

Fraction	Weight of extract	Intensity of maxima		Per cent hydrocarbon in extract	Weight of hydrocarbon in extract, in mgm.
A (hair)	237 mgm.	$E^{1\%}_{1\text{ cm.}}{}_{2,970}$	38	0.61	1.5
B (livers)	673 mgm.	$E^{1\%}_{1\text{ cm.}}{}_{2,970}$	6.2	0.10	0.7
C (skins)	18.93 gm.	$E^{1\%}_{1\text{ cm.}}{}_{2,970}$	23.6	0.38	72.0
D (carcasses)	18.65 gm.	$E^{1\%}_{1\text{ cm.}}{}_{2,970}$	8.5	0.14	26.0
E (perirenal sac and kidney)	5.43 gm.	$E^{1\%}_{1\text{ cm.}}{}_{3,485}$	1.31	0.27	14.6
Total weight of hydrocarbon recovered					114.8 mgm.
Total weight of hydrocarbon injected					320.0 mgm.
Recovery					36%

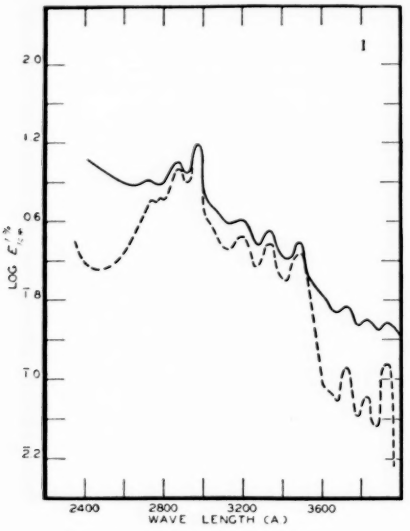


FIG. 1.—*A*, continuous line ———, neutral fraction, extract A (hair). *B*, dash line – – – –, master curve of 1,2,5,6-dibenzanthracene.

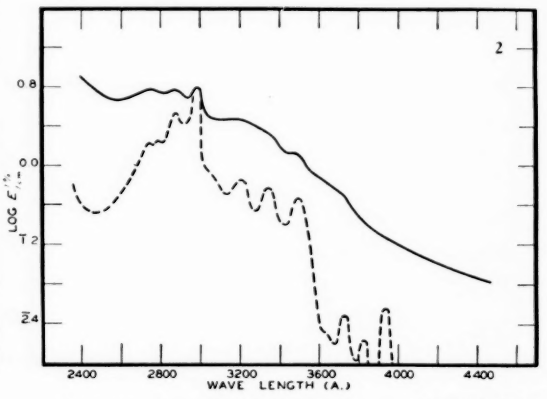


FIG. 2.—*A*, continuous line ———, neutral fraction extract B (livers). *B*, dash line – – – –, master curve of 1,2,5,6-dibenzanthracene.

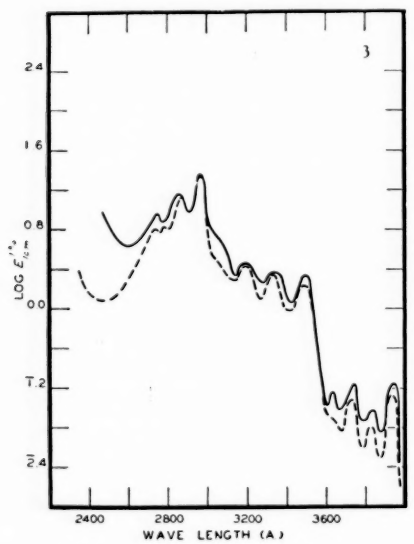


FIG. 3.—*A*, continuous line ———, neutral fraction, extract C (skins). *B*, dash line – – – –, master curve of 1,2,5,6-dibenzanthracene.

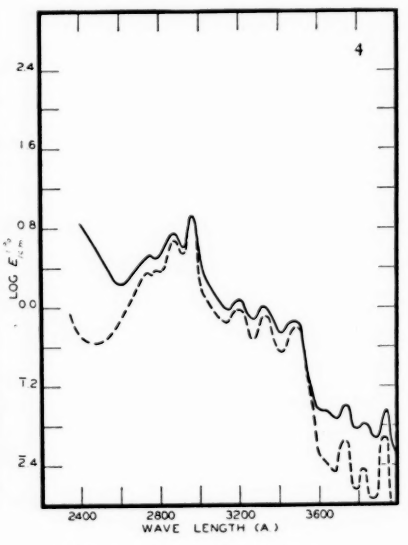


FIG. 4.—*A*, continuous line ———, neutral fraction, extract D (carcasses). *B*, dash line – – – –, master curve of 1,2,5,6-dibenzanthracene.

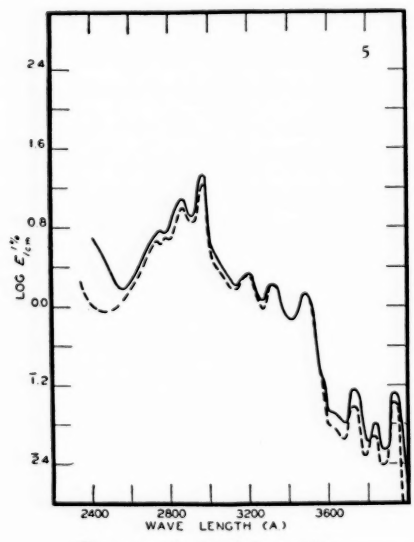


FIG. 5.—*A*, continuous line ———, neutral fraction, extract E (perirenal sac). *B*, dash line – – – –, master curve of 1,2,5,6-dibenzanthracene.

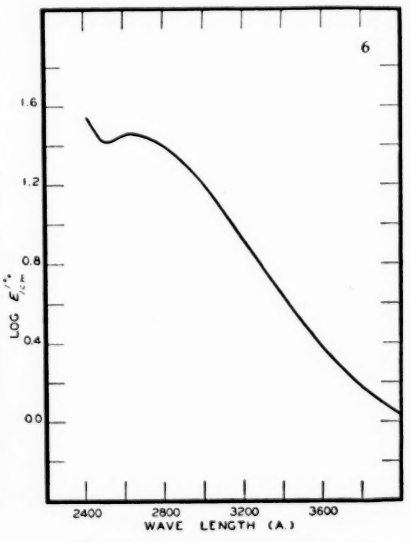


FIG. 6.—*A*, continuous line ———, nonsteam-volatile phenolic fraction of extract C (skins).

with those reported by Dobriner, Rhoads, and Lavin (1). Using a qualitative spectroscopic method these authors detected absorption bands in extracts of the intestinal contents of rats injected with dibenzanthracene, which they attributed to the unchanged hydrocarbon. No positive identification of dibenzanthracene in the liver could be obtained but extracts of whole rats gave a neutral fraction with intense hydrocarbon bands. The same authors detected absorption bands in the phenolic extract of the intestinal tract which they attributed to 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene but no similar bands could be discerned in the phenolic liver extract, while in extracts of whole rats there were faint bands which may have been due to this phenolic metabolite but were obscured by much background absorption.

DISCUSSION

Although these results cannot be quantitatively related to those discussed in the previous paper, it would appear that a significant part of the discrepancy between the amount of dibenzanthracene injected and that recoverable in the excreta may be due to the encapsulation of the hydrocarbon in vesicles near the site of subcutaneous injection, from which it may be removed only extremely slowly. Qualitative experiments [2] suggest that intraperitoneal injection of the hydrocarbon in solution in tricaprylin gives rise to considerably less local accumulation than subcutaneous injections in this or other solvents. Quantitative experiments to test this are at present in progress.

[2] I wish to thank Miss M. G. Lewisohn for assistance with this part of the work.—Author.

SUMMARY AND CONCLUSIONS

Spectrographic analysis of the organs of rats which had received subcutaneous injections of 1,2,5,6-dibenzanthracene in olive oil showed that considerable local storage of the hydrocarbon takes place in vesicles formed near the site of injection. Altogether some 36 per cent of the amount of hydrocarbon injected could be accounted for in this manner. No spectrographic evidence of the presence of the phenolic metabolite, 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene could be found in any of the tissues examined.

Qualitative experiments suggest that intraperitoneal injections of 1,2,5,6-dibenzanthracene in tricaprylin result in better absorption of the hydrocarbon than subcutaneous injections in olive oil.

The author wishes to express his appreciation of the advice and encouragement of Professor L. F. Fieser during the progress of this work and to thank Dr. C. E. Dunlap of the Collis P. Huntington Memorial Hospital, Boston, for carrying out the autopsies.

REFERENCES

1. Dobriner, K., C. P. Rhoads, and G. I. Lavin. The Spectroscopic Study of Biological Extracts. II. The Detection, Isolation, and Biological Effects of the Metabolites of 1,2,5,6-Dibenzanthracene. Cancer Research, **2**:95-107. 1942.
2. Hershberg, E. B., and J. K. Wolfe. A Rapid Extractor for Urinary Steroids. II. Modifications for the Simultaneous Hydrolysis and Extraction of Urine with any Solvent Heavier than Water. J. Biol. Chem., **141**:215-218. 1941.
3. Jones, R. N. The Spectrographic Analysis of Carcinogenic Hydrocarbons and Metabolites. I. Introduction. Cancer Research, **2**:237-244. 1942.
4. Jones, R. N. The Spectrographic Analysis of Carcinogenic Hydrocarbons and Metabolites. II. Determination of 1,2,5,6-Dibenzanthracene and 4′,8′-Dihydroxy-1,2,5,6-dibenzanthracene in Rat Excreta. Cancer Research, **2**: 245-251. 1942.

Visible Light and Skin Tumors Induced with Benzpyrene in Mice*

John J. Morton, M.D., Ethel M. Luce-Clausen, M.D., and Earle B. Mahoney, M.D.

(From the Department of Surgery and the Department of Zoology, University of Rochester, Rochester, N. Y.)

(Received for publication December 1, 1941)

We have recently reported (11) a study of the effects of visible light on the development of skin tumors induced with benzpyrene in Swiss albino mice. Doniach and Mottram (5) coincidentally with us, published work results of which are in agreement with our findings.

In this paper we report a further study in which mice of a different strain were used, and in our discussion, have attempted to cover the studies on both strains.

EXPERIMENTAL PROCEDURE

The rooms used for our experiments have been fully described (7, 11). In this experiment we used the same fluorescent lamps, in room III, as are described in (11); *i.e.,* an illumination of 300 to 200 foot candles, and a total energy of the order of 0.1 gm./cal./cm.^{2}/min. out of which about 10 per cent is in the far infra-red and about 5 per cent of wave lengths between 3,600 A. and 4,000 A. "Blue" lamps and "daylight," of the General Electric Company, were used as before.

The mice used were the C57 black, Bar Harbor strain. The experiment was started with animals 8 to 9 weeks old. They were kept in individual cages, 40 in room I (dark) and 37 in room III (light). They were fed Purina dog chow and distilled water. Males and females were distributed equally in each room; *i.e.,* 14 male, 26 female in room I, 13 male, 24 female in room III. Ten additional unpainted controls (5 male, 5 female), treated and fed similarly, were kept in each room. All mice were weighed weekly. The experimental groups, in both rooms, were painted twice weekly, in the interscapular region with 0.5 per cent solution of 3,4-benzpyrene [1] in benzene, with a No. 8 camel's hair brush. Thirty-five paintings were given over a period of 17 weeks. The mice were observed for 60 days after this. The experiment lasted from January 2 to June 28, 1941.

RESULTS

Table I gives results analyzed in the same way as those given (11) for white mice. The table for white mice has been reproduced here for purposes of comparison.

We again noticed a marked difference in the reaction of the mice in the two rooms to the first few paintings, but in this experiment the group in the light room showed, in addition to baldness, a dermatitis, marked by intense irritation and scratching. A typical case of this is shown in Fig. 1. This dermatitis appeared to be general, not local in distribution. After 15 paintings not one of the animals in the dark room showed any skin abrasion, while 16 out of 37 animals in the light room showed ulcerative patches on flanks and hip regions from scratching. At autopsy 22 out of 37 mice in the light room showed evidence of dermatitis, while not one in the dark room showed an abraded skin. No dermatitis was observed in the unpainted controls.

The incidence of cancer, by histological examination, was 89 per cent in the dark room and 53 per cent in the light room.

The growth curves of both painted and unpainted animals showed no difference, in either group, that could be attributed to environment. Thus, judged by body weight, we could not conclude that general nutrition was a factor in the results obtained.

Tumors Found on Histological Examination

The gross and microscopic appearance of the tumors which developed in these groups of mice was essentially similar to those recorded previously (11). The usual papilloma appeared as a cornified mass which showed no tendency to invasion of the surrounding skin. Microscopically the epithelium showed marked hyperplasia but no tendency to invasion of the subcutaneous tissue. The epidermoid carcinomas had a marked tendency to ulcerate and showed gross evidence of local invasion of surrounding tissues. The cells ap-

* This investigation was aided by a grant from The Rockefeller Foundation.

[1] This dosage was chosen because it was found in the experience of Mahoney, Mider, and Morton to produce *skin cancers.* These investigators found that smaller doses produced other forms of tumor, such as cancer of the breast, leukoses, etc.—Personal communication.

peared quite anaplastic, there was obliteration of the basement membrane, mitotic figures were prominent, and the normal architecture was completely destroyed. These tumors which were ulcerated showed evidence of gross secondary infection.

In one instance an adenocarcinoma of the breast was found lying immediately beneath an epidermoid carcinoma. The sarcomas were quite anaplastic but resembled the spindle cell type. The mice developing "dermatitis" did not show any evidence of malignant degeneration in the ulcerated areas. Microscopically the lesion resembled a benign ulcer with acute and chronic inflammation.

Careful note was taken of each papilloma as it appeared. Table I shows an average of 4.05 papillomas per black mouse, as compared with 2.9 per white mouse in the dark room; 2.8 per black mouse, as compared with 2.1 white, in the light room. This however does not give the complete picture. The papillomas in the light room in both experiments were different in degree and more difficult to diagnose; they appeared later, were very small, apt to disappear, and never at any time reached the "cauliflower" size of many seen in the dark room. Fig. 2 gives the results of an analysis made of the number of papillomas and carcinomas in both experiments over the time of the experiment. We charted each papilloma, from its date of onset to the time when the mouse was autopsied or died—in some cases, in the light room group, to the time when it disappeared. We then counted the total number present in the group at 10-day intervals, to get the average number per mouse in the group. This figure multiplied by 10, for the interval between counts, and added cumulatively has been called the papilloma days per mouse. The carcinomas were analyzed in a similar way and the results in each group plotted together.

Certain facts are brought out by this method of analysis which do not appear in Table I.

Comparing the papilloma-day curves of black mice in rooms I and III it is readily seen that the shape of the two curves is different. There is a longer latent period before the appearance of the 1st papilloma, and fewer papilloma days, in room III; the same is true for white mice in both groups, although there were fewer papilloma days in white than in black mice.

A similar comparison of the curves for carcinomas shows the appearance of the first carcinoma at 105 days in the dark room (room I) and at 145 days in the light room (room III) in both experiments. This indicates a slowing up of the process under the action of light. There was, however, no correlation in individual mice between the time, after the 1st painting, of the appearance of the 1st papilloma and the appearance of the 1st carcinoma.

TABLE I: EFFECT OF LIGHT AND DARKNESS ON THE DEVELOPMENT OF SKIN TUMORS INDUCED BY 3,4-BENZPYRENE

EXPERIMENT I—SWISS ALBINO MICE

		Papillomas				Carcinomas					
	Et *	Number mice with	Total number	Number per mouse	Latent interval to 1st papilloma	Number mice with	Total number	Number per mouse	Latent interval to 1st	Interval from 1st papilloma to carcinoma	Carcinoma per cent
Room I, dark	38	37	111	2.9	111 ± 4.7 days †	30	58	1.5	144 ± 4.7 days	35 ± 3.2 days	79
Room III, light	34	29	72	2.1	121 ± 5.2 days	15	28	0.8	168 ± 4.3 days	56 ± 7.3 days	44

EXPERIMENT II—C57 BLACK MICE

		Papillomas				Carcinomas					
	Et *	Number mice with	Total number	Number per mouse	Latent interval to 1st papilloma	Number mice with	Total number	Number per mouse	Latent interval to 1st	Interval from 1st papilloma to carcinoma	Carcinoma per cent
Room I, dark	39	39	158	4.05	82.1 ± 3.6 days	35	50	1.3	147 ± 4.6	66 ± 4.6	89
Room III, light	34	33	97	2.8	109 ± 4.4 days	18	22	0.6	149 ± 3.2	39 ± 5.8	53

* Et = Effective total, number of mice that did not die before the appearance of the first papilloma.
† This and all following errors = standard errors.

DISCUSSION

It is evident from these experiments, and those of Doniach and Mottram (5) that light, in the visible part of the spectrum, has some effect on the carcinogenic activity of benzpyrene when applied to the skin. Papillomas in the light are delayed in appearance, grow slowly, tend to disappear, and never reach the

size of those observed in the dark. Early effects of painting result in certain generalized effects, such as continued baldness, intense itching, and dermatitis, which only appear in animals exposed to light. The carcinomas appear later in animals exposed to light and are less numerous than in groups of animals kept in the dark.

FIG. 1.—Mouse No. 74, from light room. Killed after 24 paintings with 0.5 per cent solution of 3,4-benzpyrene in benzene, showing dermatitis.

We suggested (11) that nutrition might be a factor, but our second experiment, in which accurate records of gains in weight were kept, does not bear this out. Our data show no significant difference in general nutrition, as judged by body weight, between the dark and light groups, either painted mice, or unpainted controls.

Lewis (10) has reported the development of photosensitivity in chick-embryo cells grown in media containing carcinogenic hydrocarbons.

Doniach and Mottram (5) have reported sensitization of the skin by light, accompanied by intense irritation and scratching, a result which was very marked in our experiments with black mice. These authors suggest that the cells which take up the hydrocarbon are damaged or killed, thus reducing the total number of potential malignant cells. They emphasize, especially in experiments where sunlight was used, a *local* dermatitis, produced at site of application of carcinogen. In our experiments, where the effects of ultraviolet radiations are excluded, the dermatitis pro-

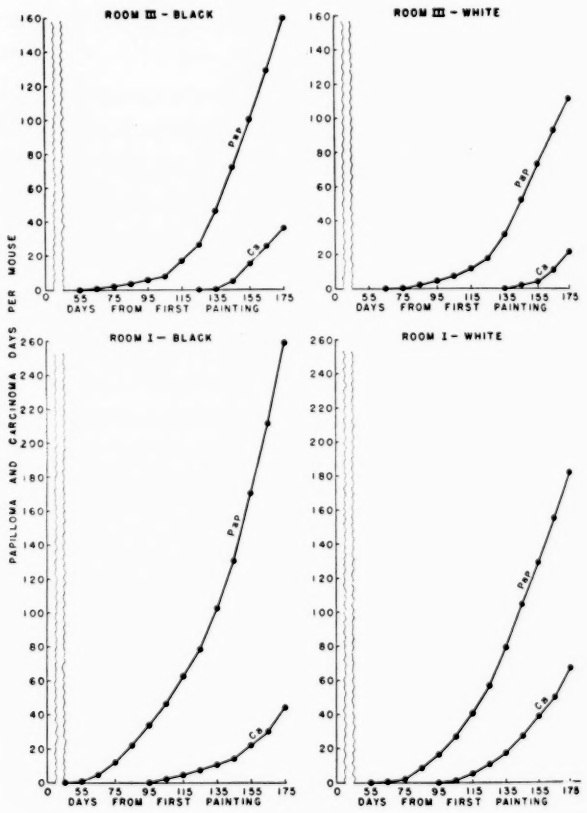


FIG. 2.—Graphs of papilloma days and carcinoma days per mouse. Room I = dark room. Room III = light room. See text for explanation of construction of graphs.

duced appeared to be general, not local, as shown in Fig. 1, with falling out of fur and presence of exudate on regions far removed from the site of painting. Certain possibilities suggest themselves when we consider the skin, under the influence of benzpyrene, as a medium for the development of tumors in light and in dark. We have no evidence in support of the idea that the carcinogen itself is changed in the presence of cells, by light, so this question must remain open. But another possibility, suggested by Charles, who arrived at it after a mathematical analysis of our data (3) is some change in the rate of growth of skin cells. This might vary with environmental conditions such as light and dark, and also with the strain of mice used.

It could be detected by histological methods involving mitotic counts.

A diurnal periodicity in mitotic division of plant and animal cells is known to exist. Karsten (8) working with *Spirogyra* noted a maximum number of mitoses at midnight, and with *Zea mais* a maximum at night, and a minimum in the day. Similar results were reported with *Allium* by Kellicott (9). Reverse effects were secured with *Pisum sativum* by Ståfelt (13).

There is scanty literature on the effects of light upon mitosis in both plant and animal cells. In 1919, Berinsohn (1) working with the roots of *Allium cepa,* showed that more cell division took place in dark than in light. These experiments were repeated in 1926 by Droogleever Fortuyn-van Leijden (6) who used fixed and stained material examined at 4-hourly intervals throughout the day and night. She found, in constant darkness, a rhythmical division with alternating maxima and minima of mitotic figures; also that radiation during the day changed the rhythm into a daily period with a maximum at night. In spite of fluctuations in the rate of mitosis in dark and light, however, she found the average percentage of nuclear divisions in both groups was the same. This would indicate that the total number of new cells, produced both in dark and in light, over a given period of time, was the same. Carleton (2) working with mice, found a 24-hourly rhythm in the mitotic divisions of skin cells, with a maximum from 8 P.M. to midnight, and a minimum about noon. She also found this periodicity destroyed by continuous exposure to light, but *not* by continuous darkness. A more recent review of the subject has been given by Cooper (4). The results, as to when the maximum and minimum counts of mitosis occur, are somewhat conflicting. Picón (12), for example, working with skin cells in mice found a maximum at noon. His work was done with very few samples. Cooper (4) on the other hand, showed that human cells (the prepuce of infants taken after circumcision) had a mean number of mitoses at night which was significantly greater than the number observed by day.

There seems to be no doubt that a diurnal rhythm exists, that it will continue in complete darkness, and that it may be modified by light.

In our experiments the radiation was not continuous, our radiated animals received alternating 12-hour periods of darkness and illumination. The illumination was fairly intense. Certain generalized effects such as dermatitis were observed in the radiated animals which were not seen in those kept in the dark.

There was, also, a higher percentage of papillomas and of tumors in the group deprived of light, and also a higher percentage of both in black than in white mice. This would indicate some difference dependent upon environment and strain. To prove that this difference was rate of growth of cells, it would be necessary to demonstrate a modification of the diurnal rhythm by light under our experimental conditions. It is possible that we are dealing with a diurnal rhythm in both groups of mice, but this rhythm may run at a higher maximum in the dark room group than in the group exposed to light. Such a theory would be in line with the prevalent idea that tumor production occurs more rapidly in well nourished animals. It must, however, mean that in a given time a larger number of skin cells are produced in darkness than in light. Such a result would run counter to the findings of Droogleever Fortuyn-van Leijden (9) for plant cells.

The possibility of gene changes governing papilloma production, has been discussed by Charles and Luce-Clausen in the paper which follows (3).

SUMMARY AND CONCLUSIONS

1. Using the C57 black, Bar Harbor, strain of mice, skin tumors were induced with 3,4-benzpyrene by painting. Two groups of mice were used; one group was kept, throughout the experiment, in an environment of visible light for 12 hours daily, the other group in complete darkness. Results similar to those reported with Swiss albino mice were obtained; namely, delay in the appearance of carcinomas, and a diminution in the number of animals which developed tumors in the group exposed to light. In addition, a marked dermatitis developed in the group exposed to light. No dermatitis was seen in unpainted controls.

2. An analysis and comparison of the results of experiments on both strains shows the appearance of the first carcinoma to be at 105 days in the dark, and at 145 days in the light, in both strains.

3. The total number of both papillomas and of carcinomas was found to be higher in the group deprived of light; but also there was a higher percentage of both in black than in white mice. This indicates a difference dependent upon both environment and strain.

4. General nutrition, as shown by differences in body weight in dark and light was found not to be a factor, in painted mice or in unpainted controls.

5. The possibility of different rates of growth of skin cells in dark and in light, in black and in white mice, has been discussed.

6. The kinetics of papilloma production is discussed by Charles in a paper which follows.

We wish to thank Dr. Donald Charles for his interest and help, and for suggesting the method of analysis which we have employed; also Miss Elizabeth Brown for skilled technical assistance.—AUTHORS.

REFERENCES

1. Berinsohn, H. W. The Influence of Light in the Cell Increase in the Roots of Allium Cepa. Proc. Sec. Sci. Amsterdam, **22**:457-461. 1919.
2. Carleton, A. A Rhythmical Periodicity in the Mitotic Division of Animal Cells. J. Anat., **68**:251-263. 1933-34.
3. Charles, D. R., and E. M. Luce-Clausen. The Kinetics of Papilloma Formation in Benzpyrene-Treated Mice. Cancer Research, **2**:261-263. 1942.
4. Cooper, Z. K. Mitotic Rhythm in Human Epidermis. J. Invest. Dermat., **2**:289-300. 1939.
5. Doniach, I., and J. C. Mottram. On the Effect of Light upon the Incidence of Tumours in Painted Mice. Am. J. Cancer, **39**:234-240. 1940.
6. Droogleever Fortuyn-van Leijden, Mrs. C. E. Day and Night Period in Nuclear Divisions. Proc. Sec. Sci. Amsterdam, **29**:Part 2, 979-988. 1926.
7. Jones, L. A., and C. Tuttle. Isolation of Desired Quantities of Radiant Energy for Biological Experimentation. J. Nutrition, **18**:611-618. 1939.
8. Karsten, G. Über Tagesperiode der Kern-u. Zellteilungen. Ztschr. f. Bot., **7**:1-34. 1915; **10**:369-388. 1918. (Quoted by Cooper.)
9. Kellicott, W. The Daily Periodicity of Cell Division and of Elongation in the Root of Allium. Bull. Torrey Bot. Club, **31**:529-550. 1904.
10. Lewis, M. R. The Photosensitivity of Chick-Embryo Cells Growing in Media Containing Certain Carcinogenic Substances. Am. J. Cancer, **25**:305-309. 1935.
11. Morton, J. J., E. M. Luce-Clausen, and E. B. Mahoney. The Effect of Visible Light on the Development of Tumors Induced by Benzpyrene in the Skin of Mice. Am. J. Roentgenol., **43**:896-898. 1940.
12. Ortiz Picón, J. M. Über Zellteilungsfrequenz und Zellteilungs-Rhythmus in der Epidermis der Maus. Ztschr. f. Zellforsch. u. mikr. Anat., **19**:488-509. 1933.
13. Ståfelt, M. G. Studien über die Periodizität d. Zellteilung u. sich daran anschliessende Erscheinungen. Kunst. Svenska Vetensk. Hand., vol. LXII:1. 1921. Quoted by Carleton (2).

The Kinetics of Papilloma Formation in Benzpyrene-Treated Mice

Donald R. Charles, Ph.D., and Ethel M. Luce-Clausen, M.D.

(From the Department of Zoology, University of Rochester, Rochester, N. Y.)

(Received for publication December 1, 1941)

When mice are painted repeatedly with benzpyrene, the consequent papillomas in the painted region do not appear simultaneously. There is an interval of 5 to 20 weeks from first painting to first papilloma, and an average interval thereafter of 3 or 4 weeks between successive papillomas, in the experiments of Morton, Luce-Clausen, and Mahoney (1). In a large group of mice, the number of papillomas rises continuously from about 5 weeks after the first painting until about a month after painting has been discontinued. The graph of total papillomas against time from beginning of treatment is like a somewhat irregular growth curve displaced considerably to the right of the time origin.

That the *step-like* accumulation of papillomas in any single mouse really depends upon some more nearly *continuous* underlying process induced in skin by benzpyrene, and that the continuous *group* curve of papilloma onset is a measure of that process, seems probable or, at least, possible. In this case the group curve is as subject to kinetic analysis as muscle or nerve responses. It is with such an attempt—tentative and incomplete—that this note is concerned. Like any kinetic analysis, it starts from more or less reasonable assumptions about the nature of the underlying processes, deduces the numerical relations that would therefore be expected in the observed data, and examines the data for these relations.

Each of the assumptions to be adopted here will be recognized as equivalent to a hypothesis of long standing and considerable debate in the literature of neoplasms. No attempt will be made to defend these particular hypotheses on their individual merits; together they have implications with which the data of papilloma onset are in fair agreement, as will be seen below. The assumptions are as follows:

1. The formation of a papilloma depends upon some self-perpetuating abnormal condition in the stratum germinativum.

2. The abnormal condition preceding any single papilloma at first involves only a single cell.

3. The first step towards the abnormal condition is mutation of some particular gene which is essential to normal differentiation of new skin cells. If normal and abnormal forms of the gene are represented by P and p, respectively, the cell is Pp at this stage in contrast to the normal cells around it which are PP. The Pp cell still gives rise to a normal lineage because it still contains one normal P. The abnormal physiology which produces a papilloma is established when the second allele mutates, so that the cell is pp. Thereafter it forms only daughter cells which become papillomatous.

4. The rate of mutation from P to p in skin cells is considerably increased by exposure to benzpyrene; in untreated skin such mutations occur *very* rarely.

Beyond these four assumptions, it may be taken as almost certain that considerable time elapses between the onset of the abnormal processes in a pp cell and the formation of a recognizable papilloma.

If all the benzpyrene paintings are done comparably, and if each painting causes a small proportion k of the P genes in the treated tissue to mutate to p, then after x paintings a proportion kx of the P genes will have mutated. And since the mutations would presumably have occurred at random, a proportion $(kx)^2$ of the cells would have undergone two, and so be pp; $2kx(1-kx)$ would have undergone only one mutation, and so be Pp and still be functioning normally; $(1-kx)^2$ would still be completely normal. In terms of time, rather than number of paintings, the proportion of pp cells is $(kt/c)^2$, t representing the time since first painting and c the interval between paintings (3.5 days in the cited work of Morton, Luce-Clausen, and Mahoney). If the painted area covers N stratum germinativum cells on the average per mouse, the number of pp cells or cell lineages per mouse after t days of treatment is $N(kt/c)^2$. Now suppose that, on the average, i days pass between the moment when a stratum germinativum cell becomes pp and the subsequent time when the tissue formed by that cell becomes recognizable as a papilloma. Then the number of papillomas to be seen t days after the first painting is the same as the number of pp cells which had existed i days previously; that is, at $(t-i)$ days after the first painting. So the average number of papillomas, n,

per mouse should be related to time from first treatment in the following way:

$$n = N[k(t-i)/c]^2$$
$$\sqrt{n} = \frac{k\sqrt{N}(t-i)}{c}$$

That is, the square root of average number of papillomas per mouse should form a straight line when plotted against time after first painting. The slope of the line, $k\sqrt{N}/c$, would be proportional to the number of mutations induced by a single painting. The time value at which the line crosses the time axis would be equal to the interval for a pp cell to form a visible papilloma.

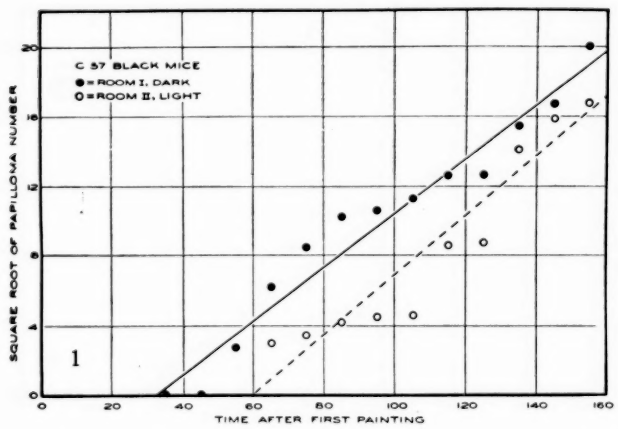
The square root of papilloma number against time is plotted in Figs. 1 and 2, for each of the four groups of benzpyrene-painted mice reported by Morton, Luce-Clausen, and Mahoney (1): strain C57 blacks and Swiss albinos, each in light and in dark (approximately 40 individuals in each group). The straight lines in Figs. 1 and 2 were fitted by the method of least squares to avoid any influence of the authors' prejudices on the slopes and intercepts obtained.

Whether or not the points lie so irregularly about the fitted lines as not really to test the assumptions on which a rectilinear relation is expected, is left to the reader's judgment. The authors feel that no complicated statistical tests are warranted, and that the agreement is about as good as could be anticipated, in view of the difficulty of getting accurate data in this sort of investigation.

In one respect, at least, what is expected under the present hypotheses about papilloma formation is found without doubt. If the slopes of the fitted lines depend upon the rate of mutation of P to p in the presence of benzpyrene, they should be equal in all four groups, which were painted comparably. Very nearly equal slopes are found: 0.0156, 0.0171, 0.0161, and 0.0163 per mouse per day for C57 mice in dark and light, Swiss albinos in dark and light, respectively. This is in agreement with the possibility suggested by Morton, Luce-Clausen, and Mahoney (1) that light may not affect the primary activity of benzpyrene.

The mutation rates which are implicit in the slopes of Figs. 1 and 2 are well within the range of rates obtained by the action of x-rays on germ cells of *Drosophila*. As seen above, slope $= k\sqrt{N}/c$; hence $k = \text{slope} \times c/\sqrt{N}$. That is, the proportion of P genes which mutate to p after each benzpyrene painting is the slope multiplied by the interval between paintings and divided by the square root of the number of stratum germinativum cells underlying the painted area. This area is about 2 cm.^{2} in the present material. It covers roughly 4,000,000 stratum germinativum cells, of 8μ average diameter parallel to the skin surface. So the presumptive mutation rate is $0.016 \times 3.5/2000$ or about 1/36000. In *Drosophila* Timoféef-Ressovsky (2) found 1/2000 of W genes (essential for formation of normal red eye pigment) to mutate to an inactive form w at an x-ray exposure of 4,800 r. Most other genes of *Drosophila* have considerably lower mutation rates, but it is interesting to note that at least one can be induced to mutate much more frequently than the assumed P gene of mouse cells under the action of benzpyrene.

Insofar as the time intercepts of Figs. 1 and 2 represent intervals in which single mutant stratum germinativum cells give rise to recognizable masses of papillomatous tissue, they permit a conclusion about skin growth rates which will be subject to test by direct observation. The intercepts are: black mice in dark,



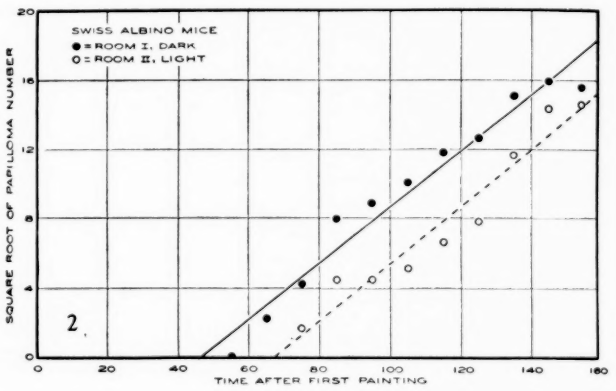


FIG. 1.—The increase in papilloma number per hundred C57 black mice, under semiweekly paintings with benzpyrene—data of Morton, Luce-Clausen, and Mahoney (1).

FIG. 2.—The increase in papilloma number per hundred Swiss albino mice, under semiweekly paintings with benzpyrene—data of Morton, Luce-Clausen, and Mahoney (1).

32.2 days; black in light, 59.8; white mice in dark, 46.0; white in light, 66.7. Thus the blacks have shorter latent intervals than the albinos, in either light or dark; and both blacks and albinos have shorter latent intervals in the dark than in the light. It seems probable that the latent interval depends primarily on the growth rate of the prospectively papillomatous tissue, which might possibly be identical with the growth rate of the surrounding normal tissue. In that case the differing latent intervals would indicate that mouse skin grows only about 60 per cent as fast, on the average, in light (of the intensity used by Morton, Luce-Clausen, and Mahoney) as in dark, and the skin of Swiss albinos only about 80 per cent as fast as the skin of C57 blacks under comparable conditions. Such differences may perhaps be detectable by counts of mitotic frequency in the stratum germinativum.

In summary, the accumulation of papillomas in benzpyrene-treated mice may be pictured as follows, with a degree of validity which depends upon the extent to which future observations may confirm and extend the present tests:

At each painting, as done by Morton, Luce-Clausen, and Mahoney (1), benzpyrene diffuses into roughly 4,000,000 stratum germinativum cells per mouse. After the first painting about 3,999,800 of the cells remain unaffected, but in roughly 200, one of the two P genes mutates to p. These 200 Pp cells continue to function normally because they still have one normal P. Each subsequent painting causes more PP cells to become Pp (1/18000 on the average) and as Pp cells accumulate, the chance increases that one of them will be converted by a second mutation into pp (the chance is 1/36000 that any single Pp cell will be so affected by a single painting). Once a cell has become pp its subsequent progeny differentiate abnormally, forming a papilloma which is recognizable about 32, 46, 60, or 68 days later depending on the strain and illumination of the mouse in which the pp cell lies. After 12 paintings (6 weeks) one in every 3,000 original P genes of the treated area has become p. The mutations, occurring at random, have converted about 2,700 cells into Pp, and perhaps one or two into pp. Two pp cells would be expected at this time in about 8 per cent of treated mice; one pp cell, in roughly 28 per cent; and none, in the remainder. So, if the mice are Swiss albinos and they are kept in the dark, about 64 per cent of them will still be unaffected, 28 per cent will have a single papilloma, and 8 per cent will have 2 papillomas, 46 days after the 12th painting; *i.e.,* after 88 days of the experiment. The mean number of papillomas per hundred mice will be $28\times1+8\times2$ or 44; its square root is 6.6, in agreement with Fig. 2. By similar calculations one could reconstruct all four fitted lines.

Finally, the fact that the course of increase in papilloma number among benzpyrene-treated mice can be roughly synthesized in terms of a mutation rate, a cell number, and a growth interval is still far from proving that mutations really are involved. Some quite different hypothesis might perhaps have the same numerical consequences.

REFERENCES

1. Morton, J. J., E. M. Luce-Clausen, and E. B. Mahoney. Visible Light and Skin Tumors Induced With Benzpyrene in Mice. Cancer Research, **2**:256-260. 1942.
2. Timoféef-Ressovsky, N. W. The Experimental Production of Mutations. Biol. Rev., **9**:411-457. 1934.

The Lack of Influence of Di-(Hydroxymethyl) Peroxide on the Incidence and Growth of Transplanted, Induced, and Spontaneous Mouse Tumors*

I. Transplanted Tumors

Morris Belkin, Ph.D.

(From the Department of Pathology, Yale University School of Medicine, New Haven, Conn.)

(Received for publication January 7, 1942)

In a number of papers Maisin and his collaborators (3-6) have reported a series of experiments on the effect of organic peroxides on the prevention of benzpyrene-induced cancer in mice.

The primary thesis upon which these investigations were based and their results may be summarized as follows:

The researches of Warburg have shown that malignant tissues have a greater anaerobic glycolysis than corresponding normal tissues, suggesting a disturbance of an oxidative enzyme. Carcinogenic substances have been shown to have an inhibiting action on normal oxidation processes of cells *in vitro*. It seemed likely, therefore, that in carcinogenesis the process of cellular oxidation plays a primary role. Hence the use of substances which readily yield oxygen as antiblastic agents seemed logical. To this end, organic peroxides and peroxide-forming substances ("peroxydogènes"), such as performic acid and especially "diperoxide of diformaldehyde," were used.

Maisin *et al.* (3-6) have reported that the use of such peroxides has resulted in a decreased cancer incidence in benzpyrene-treated mice receiving these substances. Administration of the material was made either in aqueous solution or in the form of crystals. They emphasize the point that one injection was just as efficacious as repeated doses in securing the desired effect, since it is a phenomenon of catalytic action of long duration, whatever the nature of this action may be.

Also, there was a different effect on cancer incidence depending upon the concentrations used. Strong concentrations; *i. e.*, up to 1×10^{-3}, had an activating or stimulating effect, hastening the appearance and increasing the incidence of malignant growths. Beginning with a dilution of 1×10^{-5} an inhibitory effect makes itself felt, and this inhibition increases up to a dilution of 1×10^{-18}. This last dilution exerts the strongest preventive action [1] although repeated injections may be necessary.

In May, 1938, a comprehensive series of experiments was begun in the laboratories of the Department of Pathology at Yale University School of Medicine, to repeat and amplify the above-reported observations on the prophylactic effect of "diperoxide of diformaldehyde" on malignant growths. The following report will be concerned with the preparation and use of this compound and the results of its administration in experiments with mouse tumors. As will be indicated later, some of the experiments to be reported were set up under the direction of Doctor Maisin.

PREPARATION OF DI-(HYDROXYMETHYL) PEROXIDE AND DILUTIONS USED

Under Doctor Maisin's personal supervision the following procedure was worked out by him and Dr. Julius White for obtaining di-(hydroxymethyl) peroxide [2] (the diperoxide of diformaldehyde of Maisin), used in the experiments reported in this communication.

Eighty cc. of ether (U. S. P.) were measured into each of two clean, dry, brown bottles. Into one bottle was added 20 cc. of 36 per cent formaldehyde (Merck); into the other 20 cc. of super-oxol (Merck—30 per cent H_2O_2). The bottles were stoppered, shaken thoroughly for a few minutes, and placed in a dark cupboard at room temperature for 24 to 48 hours. After this interval, the top ether-formaldehyde layer in one case and the top ether-peroxide layer in the other were poured off and placed in two other brown bottles, each of which contained about ¾ inch anhydrous sodium sulfate. They were placed in a dark cupboard for 24 to 48 hours at room temperature.

The dried ether-formaldehyde layer and the ether-peroxide layer were then decanted into each of two clean containers. Four cc. of the ether-formaldehyde solution were then pipetted into each of a desired

* This investigation was aided by a grant from The Hubbard-McCormick Clinical Cancer Research Fund.

[1] Personal communication.

[2] Hereafter referred to simply as peroxide.

number of accurately weighed bottles. To each of these containers was added 2.2 cc. of the ether-peroxide solution. The weighing bottles were then placed in a vacuum desiccator over C. P. sulfuric acid, the desiccator evacuated for about one minute and placed in the refrigerator. In about 2 days all the ether evaporated, leaving a colorless oil which then crystallized into white prisms in about 48 hours more. This crystalline material was found to have the formula of $HOCH_2 \cdot O \cdot O \cdot CH_2OH$ and melted at 62 to 64° C. Other chemical and physiological properties were reported in a paper by White and Winternitz (8).

To make solutions for injection, crystals of peroxide were dissolved in sufficient distilled water (3 times distilled) so that each cc. contained 4 mgm. Twenty-five cc. of this stock solution plus 75 cc. distilled water gave 100 cc. of a solution of 1×10^{-3}. Greater dilutions were made by addition of appropriate amounts of distilled water to requisite amounts of the 1×10^{-3} dilution.

Pyrex glassware was used throughout and most meticulous precautions were employed to insure utmost cleanliness of all apparatus used. Syringes, bottles, and pipettes were treated as follows: After soaking in cleaning fluid for 24 hours, they were rinsed thoroughly with tap water, then filled and emptied 50 times with tap water, 50 times with once-distilled water, and 50 times with 3 times distilled water. Needles were first soaked in ether (C. P.) for 24 hours, then in 95 per cent alcohol for 24 hours, then filled and emptied with tap and distilled waters as described above.

Doctor White was concerned with the preparation of the peroxide and necessary dilutions until September, 1939, after which the writer continued until the end of the investigation.

TRANSPLANTED TUMORS

Eight hundred mice of the ABC albino strain were injected subcutaneously in the right flank with 0.1 cc. of a suspension of tumor 15091a. This is a rapidly growing neoplasm which arose as a spontaneous mammary carcinoma in an A strain mouse, as reported by Cloudman (2) and which grows in the ABC albino in a very high percentage of cases. The tumor and the ABC albino mice were originally obtained from the Roscoe B. Jackson Memorial Laboratory, and the progeny of these mice used in this experiment were raised in this laboratory. They were from 2 to 3 months old at the time of injection and were distributed proportionately among the experimental and control groups with regard to age and sex. They were kept in groups of 4 in pyrex glass jars with wire mesh covers, the jars being sterilized weekly; and received a diet of oats and Purina fox chow. Unlimited tap water was always available.

The suspension (7) was prepared by passing pieces of tumor, dissected free from necrotic material, through a Latapie mincing machine and adding 10 cc. physiological saline containing 10 per cent gelatin for each gm. of minced tissue. Further dispersion of the minced tissue was effected by gentle shaking with sterile glass beads for 20 minutes. The coarser particles were then removed by slow centrifugation for 2 minutes and this stock suspension (1:10) was diluted to 1:100 by addition of appropriate amounts of the salt-gelatin solution. This 1:100 suspension was the one used for injection.

Since with all possible precautions tumor cells *in vitro* become progressively attenuated, it was deemed desirable to use a suspension for not more than 2 hours after the tumor was first minced. This necessitated the use of two different tumor preparations, but by mixing the mice receiving injections from each suspension among all the experimental and control groups a representative distribution of animals from both tumor preparations was obtained in each group.

The mice were divided into 8 groups of 100, each group receiving the following treatment: Group 1, di-(hydroxymethyl) peroxide, concentration 1×10^{-3}; group 2, di-(hydroxymethyl) peroxide, concentration 1×10^{-9}; group 3, di-(hydroxymethyl) peroxide, concentration 1×10^{-18}; group 4, controls, distilled water; groups 5, 6, 7, and 8, controls, untreated.

The animals in each of the first 4 groups received injections every 2 weeks of 0.5 cc. of their respective peroxide concentrations, or of distilled water. The injections were always made subcutaneously at a distance from the tumor. The tumor was inoculated on September 18, 1938; the peroxide injections were begun the following day and continued till the death of each animal.

Beginning on the 16th day after inoculation, the mice were examined at weekly intervals for appearance and size of tumor. The growths were measured along their three diameters with a pair of calipers and the readings expressed in millimeters. Throughout this experiment the average of the three diameters is used to denote the size of the tumors.

The total number of tumors which arose in each group is given in Table I.

Inspection of the data in Table I shows that the maximum difference between the lowest tumor incidence (group 8) and the highest tumor incidence (groups 2 and 6) is 6 per cent, and that there is no indication that peroxide administration either stimulated or retarded the appearance of tumors in any of the experimental groups as compared with the controls.

As can be seen from the last column in this table, a few mice in each group did not develop a tumor,

although these mice were kept for 6 months after inoculation. The reason may be twofold: 1. about 0.5 per cent of mice of this strain appear to be naturally resistant to the tumor used as shown by Bunting *et al.* (1), and 2. the concentration of the inoculum employed was as low as possible so as to prolong the latent period of tumor onset and hence prolong the life of the mice. Under these circumstances some injections may have contained but few viable cells which for various reasons did not grow. It is felt that both these factors operated under these experimental conditions to give the negative results in each group.

During the first 50 days after inoculation, tumors appeared at about the same rate in each group. At about 20 days after inoculation half the mice had growths whose mean diameter was 5 mm. or greater. A very few tumors did not reach these dimensions until later. The rate of appearance of the tumors (average diameter 5 mm. or more) in each group is shown in Figs. 1 and 2. Here the ordinate indicates the number of tumor-bearing mice expressed as per cent of the effective total, and the abscissa the number of days after inoculation. For convenience the 8 groups have been divided into 2 subgroups of 4: Fig. 1 showing tumor appearance rate among the groups receiving peroxide and distilled water and Fig. 2 comprising the untreated controls.

Inspection of the two sets of curves shows marked similarity as to their shape and slope. The differences are due to biological variability normally to be found in such a tumor population. The curves of any of the peroxide-treated groups can be matched by a control group within the limits of error to be encountered under these experimental conditions.

The rate at which the tumors grew in each group is illustrated in Figs. 3 and 4. Here the ordinate is the mean size of the tumors expressed in millimeters, the abscissa is the number of days after each tumor was first noted. Hence, the time intervals at which growth was measured are the same for each tumor till the death of its host. Examination of the curves shows that the tumors in all the groups grew at the same rate from the beginning to the end of the experiment. Again a curve depicting growth rate of any group receiving peroxide can be closely paralleled by a control group within the experimental error inherent in the method of measurement employed. This was found to be ± 15 per cent. The skin thickness was not taken into consideration; this would introduce an error greater for smaller tumors than larger ones. Some of the control curves indicate growth at a somewhat slower rate than any group receiving peroxide. This, however, is not considered significant but within the error of the experimental conditions.

Another measure of tumor growth is the weight of the tumor at the death of its host. The range in such terminal weights of the tumors is shown in Figs. 5 and 6. Considering the difficulties under which such data are gathered; *i. e.*, autolysis, water loss, etc., the correlation among the groups is surprisingly good. Again, as expressed by tumor weight at death, there was no significant difference in growth rate between any of the experimental and control groups.

TABLE I: NUMBER OF TUMORS APPEARING IN EACH GROUP

Group	Number of mice (effective total)	Number of tumors	Per cent of tumors *	Mice without tumors
1. Peroxide 1×10^{-2}	97	90	94	7
2. Peroxide 1×10^{-3}	99	94	95	5
3. Peroxide $1 \times 10^{-3.5}$	100	93	93	7
4. Control—distilled water	99	91	92	8
5. Control—untreated	100	94	94	6
6. Control—untreated	99	94	95	5
7. Control—untreated	100	94	94	6
8. Control—untreated	100	89	89	11

* Per cent of effective total mice.

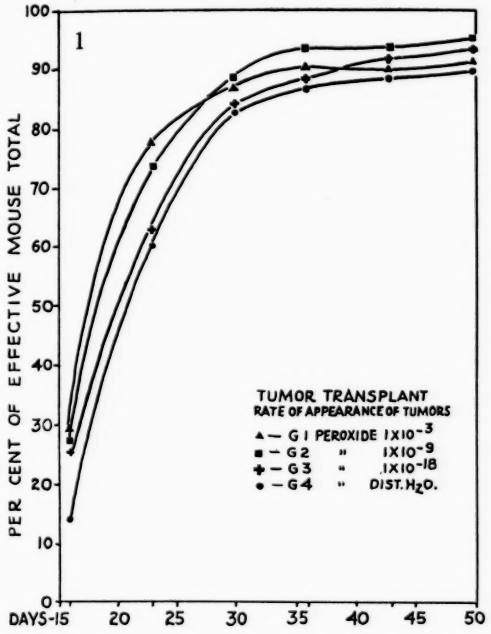
GROSS AND MICROSCOPIC FINDINGS

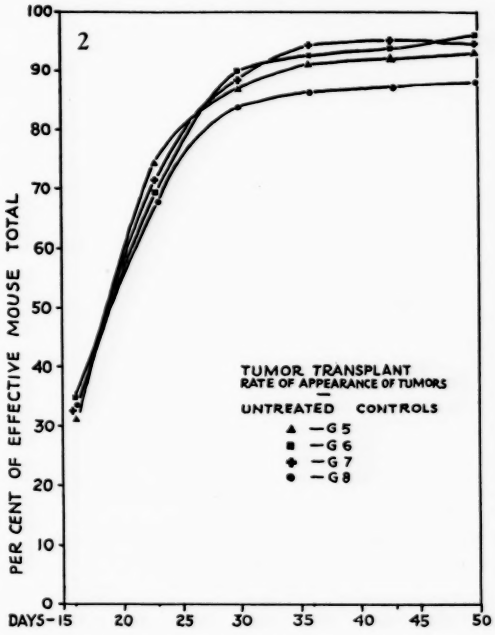
At autopsy the tumors grossly displayed considerable variation among all the groups from cases with abundance of healthy tissue with relatively little necrosis to those exhibiting massive necrosis and hemorrhage. As a general rule the smaller the tumor, the more viable tissue and the less necrosis. No correlation whatever was noted between tumor appearance and peroxide administration. Tumors of these groups receiving the different concentrations of this substance showed the same picture and to the same degree of variability as the distilled water and untreated controls.

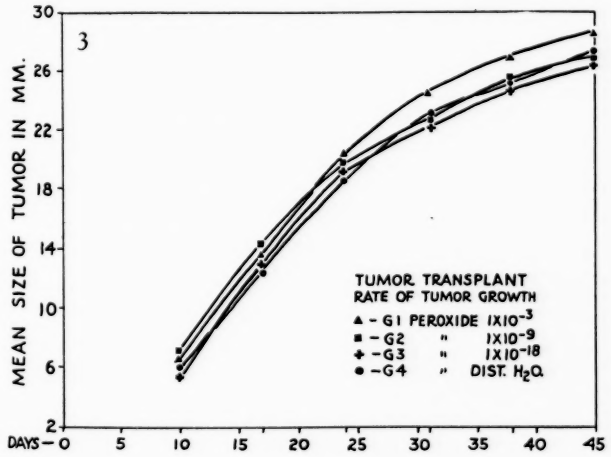
On section, the tumors presented the same microscopic picture of medullary carcinoma as that of the parent tumor used for inoculation. Mitotic figures were variable in number. Necrosis and hemorrhage were the rule, but their extent could better be determined from gross inspection of a tumor than from one or two sections of it.

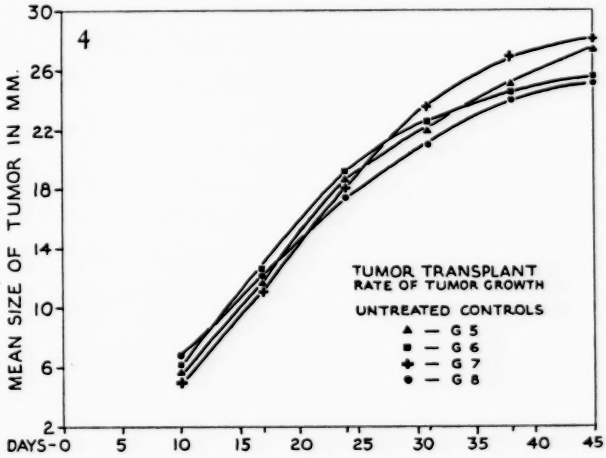
SUMMARY AND CONCLUSIONS

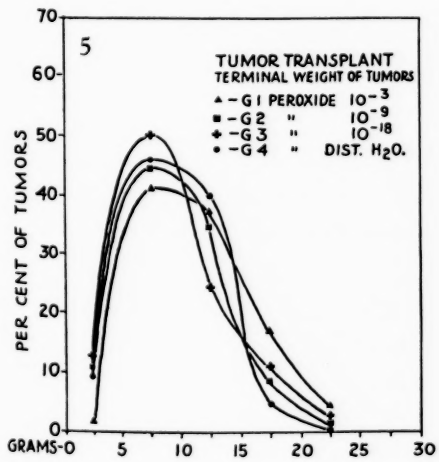
Eight hundred mice of the ABC albino strain were inoculated with tumor 15091a, a mammary carcinoma

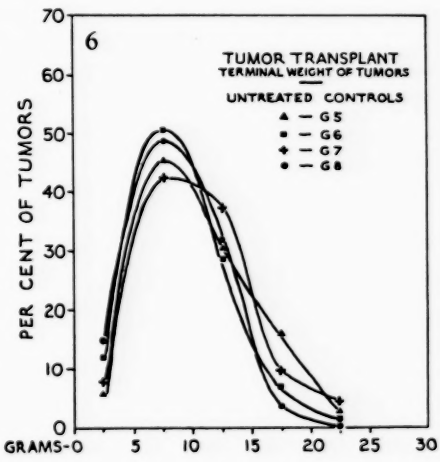

1
PER CENT OF EFFECTIVE MOUSE TOTAL
DAYS-15 20 25 30 35 40 45 50
TUMOR TRANSPLANT
RATE OF APPEARANCE OF TUMORS
▲ — G 1 PEROXIDE 1X10-3
■ — G 2 " 1X10-9
✚ — G 3 " 1X10-18
● — G 4 " DIST. H2O.


2
PER CENT OF EFFECTIVE MOUSE TOTAL
DAYS-15 20 25 30 35 40 45 50
TUMOR TRANSPLANT
RATE OF APPEARANCE OF TUMORS
UNTREATED CONTROLS
▲ — G 5
■ — G 6
✚ — G 7
● — G 8


3
MEAN SIZE OF TUMOR IN MM.
DAYS-0 5 10 15 20 25 30 35 40 45
TUMOR TRANSPLANT
RATE OF TUMOR GROWTH
▲ - G 1 PEROXIDE 1X10-3
■ - G 2 " 1X10-9
✚ - G 3 " 1X10-18
● - G 4 " DIST. H2O.


4
MEAN SIZE OF TUMOR IN MM.
DAYS-0 5 10 15 20 25 30 35 40 45
TUMOR TRANSPLANT
RATE OF TUMOR GROWTH
UNTREATED CONTROLS
▲ — G 5
■ — G 6
✚ — G 7
● — G 8


5
PER CENT OF TUMORS
GRAMS-0 5 10 15 20 25 30
TUMOR TRANSPLANT
TERMINAL WEIGHT OF TUMORS
▲ - G 1 PEROXIDE 10-3
■ - G 2 " 10-9
✚ - G 3 " 10-18
● - G 4 " DIST. H2O.


6
PER CENT OF TUMORS
GRAMS-0 5 10 15 20 25 30
TUMOR TRANSPLANT
TERMINAL WEIGHT OF TUMORS
UNTREATED CONTROLS
▲ — G 5
■ — G 6
✚ — G 7
● — G 8

FIGS. 1 TO 6

that grows rapidly in this strain. Some of the mice received bi-weekly injections of aqueous solutions of di-(hydroxymethyl) peroxide in various concentrations, some received distilled water, and others acted as untreated controls.

From the standpoint of tumor incidence, rate of tumor growth, and terminal weight of tumors, no effect of peroxide administration was discernible, either stimulating or inhibitory, as has been reported by Maisin and his collaborators.

It is a pleasure to acknowledge the technical assistance of Miss Janice C. Humphrey and Mr. Bernard S. Meyrowitz.—Author.

REFERENCES

1. Bunting, H., L. L. Waters, and H. Ryder. Studies on the Incidence and Development of Tumor 15091a in Susceptible and Resistant Strains of Mice. Yale J. Biol. & Med., **13**:387-396. 1941.
2. Cloudman, A. M. A Comparative Study of Transplantability of Eight Mammary Gland Tumors Arising in Inbred Mice. Am. J. Cancer, **16**:568-630. 1932.
3. Koch, W. F., and J. Maisin. Influence des peroxydes organiques sur la prophylaxie du cancer expérimental de la souris. Compt. rend soc. de Biol., **120**:106-108. 1935.
4. Maisin, J., and F. Robert. La prophylaxie du cancer au benzène pyrène par les peroxydes organiques. Compt. rend. soc. de Biol., **123**:156-159. 1936.
5. Maisin, J., and Y. Pourbaix. Cancer expérimental au benzopyrène et peroxydes organiques. Commun. Second Internat. Cancer Congress, **II-III**:24-30. 1937.
6. Maisin, J., Y. Pourbaix, and P. Caeymaex. L'influence de très faibles doses de peroxyde de diformaldéhyde sur l'éclosion du cancer au benzopyrène de la souris. Compt. rend. soc. de Biol., **127**:1479-1481. 1938.
7. Shear, M. J. Chemical Studies on Tumor Tissue. III. Titration of Mouse Tumors. Pub. Health Rep., **51**:668-676. 1936.
8. White, J., and M. C. Winternitz. A Study of the Behavior of Di-(Hydroxymethyl) Peroxide *in vivo* and *in vitro* and its Effect on the Catalase Activity of the Blood. Am. J. Cancer, **36**:269-275. 1939.

The Lack of Influence of Di-(Hydroxymethyl) Peroxide on the Incidence and Growth of Transplanted, Induced, and Spontaneous Mouse Tumors*

II. Tumors Induced by Cutaneous Painting with Benzpyrene

III. Tumors Induced by Subcutaneous Injection of Benzpyrene

Morris Belkin, Ph.D.

(From the Department of Pathology, Yale University School of Medicine, New Haven, Conn.)

(Received for publication January 7, 1942)

In a previous communication (1) it was shown that the biweekly injection of an aqueous solution of di-(hydroxymethyl) peroxide had no discernible effect on the incidence and rate of growth of the mammary tumor 15091a transplanted in mice of the ABC albino strain.

The present report is concerned with experiments in which this substance was administered to mice, some of which were painted cutaneously with benzpyrene and in some of which benzpyrene was injected subcutaneously. The preparation of the di-(hydroxymethyl) peroxide,[1] the diet, and housing conditions of the animals were identical with those described in the experiments dealing with transplanted tumors (1). In this paper there is also given a more detailed discussion of the reported prophylactic action of this substance as described by Maisin and his collaborators,[2] with regard to its cancer-preventing action in mice painted with benzpyrene.

II. Tumors Induced by Cutaneous Painting with Benzpyrene

Series 1

Four hundred mice of the Bagg albino strain were divided into 4 groups of 100 each, distributed proportionately with regard to age and sex. The mice were secured from the Roscoe B. Jackson Memorial Laboratory and were 8 to 10 weeks old at the start of the experiment.

Three times a week, on alternate days, 2 drops of a 0.5 per cent solution of benzpyrene (obtained from Meurice, Brussels), dissolved in C. P. benzene (thiophene-free) were applied to the interscapular area of each mouse by means of a dropping pipette. Twenty-five such applications were made in all, after which the mice received no further benzpyrene treatment. Since the pipettes delivered 25 drops per cc., the total of 50 drops of the 0.5 per cent solution of benzpyrene yielded 10 mgm. of the carcinogen applied to each mouse.

The four groups of mice received the following treatment: Group 1, peroxide 1×10^{-4}; group 2, peroxide 1×10^{-10}; group 3, peroxide 1×10^{-19}; group 4, controls, untreated.

Each of the first three groups received subcutaneously 0.5 cc. of an aqueous solution of their respective peroxide concentrations every 2 weeks. The paintings with the carcinogen were begun May 13, 1938. The peroxide injections started the following day, each animal receiving biweekly injections till death.

Beginning one month after benzpyrene painting was begun, the mice were examined for the presence of papillomas. Such surveys were made every 10 days for the first 9 months and then every 20 days until the conclusion of the experiment. This experiment was set up under the personal direction of Dr. J. Maisin, who also participated in the majority of the benzpyrene applications, the peroxide injections for the first 6 weeks, and took part in the first examination for papillomas.

Table I gives the figures for the total number of papillomas found in each group.

After about 2 months following the onset of painting, some of the papillomas assumed characteristics which were judged grossly to be malignant. In this connection, certain criteria for malignancy had to be

* This investigation was aided by a grant from The Hubbard-McCormick Clinical Cancer Research Fund.

[1] Hereafter referred to as peroxide.

[2] For references to work of Dr. Maisin and collaborators see (1).

decided upon. Mere increase in size, even though at times such increase was considerable, was not considered sufficient gross evidence upon which to base a diagnosis of malignancy. Although size was considered to some extent, invasiveness was held to be a much more critical characteristic, and diagnosis of gross malignancy was not made until signs of infiltration into the adjacent or underlying tissues were grossly malignant in the same groups. When the histopathological diagnosis of the tumors was completed, it was evident that the gross diagnosis for malignancy was lower in every group than the microscopic, the greatest error occurring among the smaller tumors.

It is held by Maisin [3] that peroxide administration should be just as effective in the prevention of any sort of induced or spontaneous tumor, regardless of

TABLE I: INCIDENCE OF PAPILLOMAS (SERIES 1)

Group	Number of mice at start	Effective total	Number of papillomas	Per cent papillomas (Per cent of effective total)
1. Peroxide 1×10^{-4}	100	100	68	68
2. Peroxide 1×10^{-10}	100	94	57	61
3. Peroxide 1×10^{-19}	100	93	80	86
4. Control—untreated	100	93	65	71

TABLE II: INCIDENCE OF GROSSLY MALIGNANT TUMORS (SERIES 1)

Group	Number of mice at start	Effective total	Number grossly malignant	Per cent of effective total grossly malignant
1. Peroxide 1×10^{-4}	100	100	51	51
2. Peroxide 1×10^{-10}	100	92	35	38
3. Peroxide 1×10^{-19}	100	92	63	69
4. Control—untreated	100	92	54	59

TABLE III: INCIDENCE OF VARIOUS TYPES OF MALIGNANT TUMORS IN BENZPYRENE-PAINTED MICE (SERIES 1) *

Group	Effective mouse total	Epidermoid carcinoma		Lung cancer		Breast cancer		Other types of tumor	Total, per cent
		Number of tumors	Per cent	Number of tumors	Per cent	Number of tumors	Per cent		
1. Peroxide 1×10^{-4}	100	56	56	13	13	3	3	1 Fibrosarcoma, 1 per cent 1 Lymphosarcoma, 1 per cent 1 Rhabdomyosarcoma, 1 per cent	75
2. Peroxide 1×10^{-10}	92	51	55	17	18	5	5	1 Squamous cell carcinoma of intestine, 1 per cent	79
3. Peroxide 1×10^{-19}	92	71	78	21	23	2	2	2 Fibrosarcoma, 2 per cent 1 Squamous cell carcinoma of stomach, 1 per cent	103
4. Controls—untreated	92	58	63	18	20	10	11	1 Squamous cell carcinoma of stomach, 1 per cent	95

* Based on microscopic examination. Percentages calculated from effective total mice for each group.

evident. Although gross diagnosis is necessarily subject to error in judgment, it was deemed of interest to make such diagnosis with regard to appearance of malignancy among the various groups with respect to time.

The total numbers of tumors in each group judged to be malignant are given in Table II.

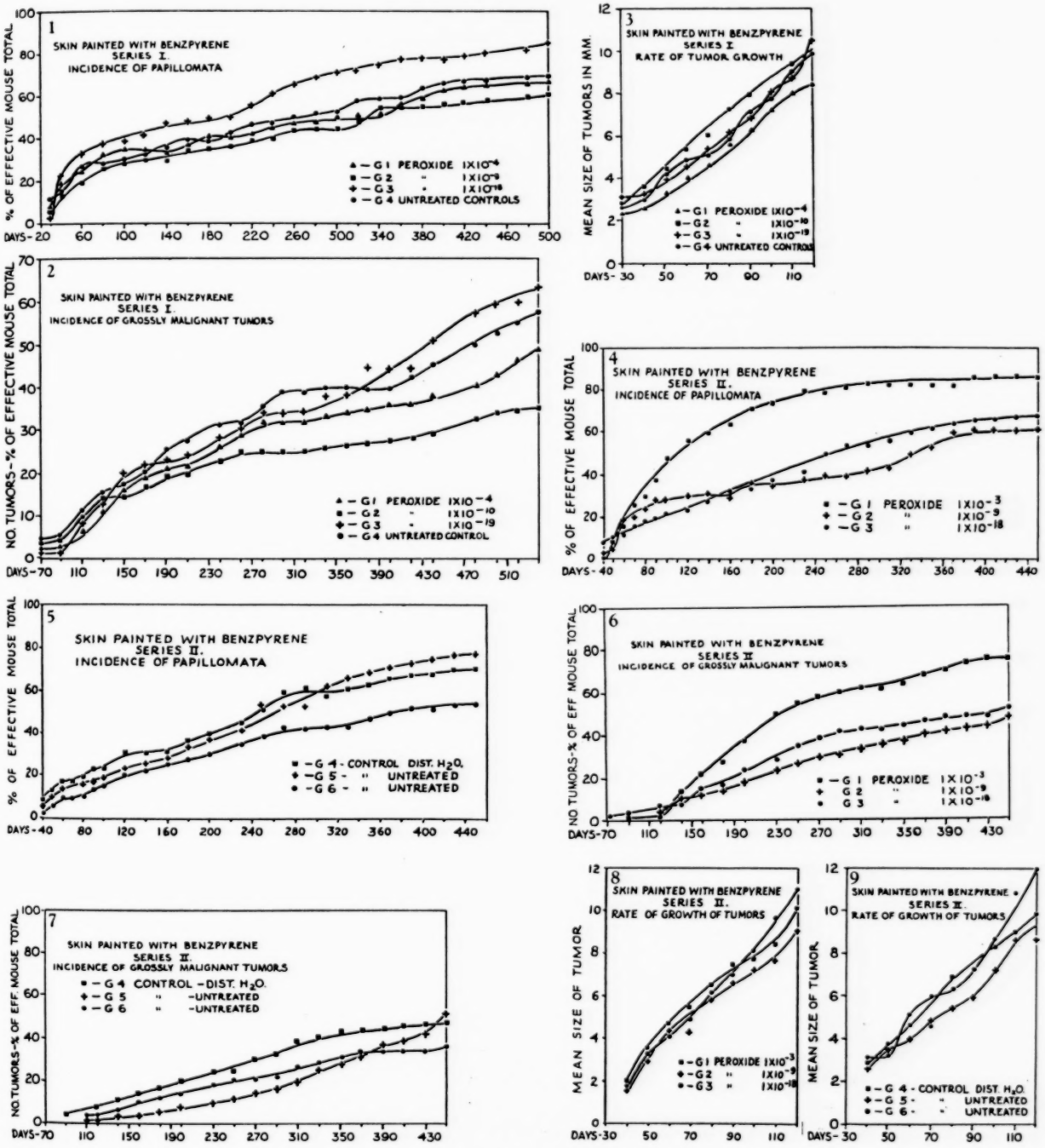
In Fig. 1 is graphically shown the rate of papilloma incidence for the experimental and control groups; and in Fig. 2, the incidence of tumors judged to be tissue origin. Although in this experiment interest was primarily focused on the production of skin cancer, other types of tumors were also encountered. In Table III the number and different kinds of tumors found, based on histologic diagnosis, are presented. The percentages of the various types of tumors obtained are in each group calculated from the effective mouse total for that group.

[3] Personal communication.

The progressive rate of growth of the tumors was followed by measuring their three diameters, and using the mean of these measurements as a single expression of their size.

Series 2

In this series, 300 Bagg albino mice were divided into 6 groups of 50 each. These mice were the progeny of parents originally secured from the Roscoe B. Jackson



Figs. 1 to 9

In Fig. 3, the rate of tumor growth among the groups is depicted. It can be seen that the shape and slope of the curves are very similar, and within the error of the method of measurement which is about ±15 per cent.

Memorial Laboratory and were raised in this laboratory. The mice were 8 to 10 weeks old at the start of the experiment and were distributed as evenly as possible with regard to sex and age. The six groups and the treatment given each were as follows: group 1,

peroxide 1×10^{-3}; group 2, peroxide 1×10^{-9}; group 3, peroxide 1×10^{-18}; group 4, distilled water; group 5, untreated controls; group 6, untreated controls.

The first four groups received, every 2 weeks, 0.5 cc. of an aqueous solution of their respective peroxide concentrations or of distilled water; the last two groups served as untreated controls. The mice in this series had the hair in the interscapular area clipped 2 days before benzpyrene application was begun; and, as can be noted, the peroxide concentrations in each group are 10 times as strong as in the corresponding group of the experiment reported above (series 1). This was done to make the range of peroxide concentrations used in this instance the same as reported by Maisin. In every other respect, however, this experiment was conducted precisely as was the former. It was begun March 15, 1939, and was terminated after 450 days.

At the end of that period, the total number of papillomas obtained in each group is given in Table IV.

Employing the same criteria for macroscopic evidence of malignancy described for the previous experiment, the number of malignant tumors obtained in each group at the close of the experiment is given in Table V.

As in the previous experiment (series 1) the incidence of benign papillomas and grossly malignant tumors was plotted with respect to time. The results are given in Figs. 4 and 5 for the papillomas and in Figs. 6 and 7 for the tumors considered grossly malignant. It is apparent that these curves are comparable in their shape and slope and show no significant difference in tumor appearance rate. As before, histologic diagnosis of the tumors indicated that the gross diagnosis of malignancy resulted in lower values than were found by microscopic examination. The number and various types of tumors obtained, based on histologic diagnosis, are given in Table VI.

The rate of growth of the tumors is graphically

TABLE IV: INCIDENCE OF PAPILLOMAS (SERIES 2)

Group	Number of mice at start	Effective total	Number of papillomas	Per cent papillomas (Per cent of effective total)
1. Peroxide 1×10^{-3}	50	50	47	94
2. Peroxide 1×10^{-9}	50	49	33	67
3. Peroxide 1×10^{-18}	50	49	34	69
4. Control—distilled water	50	49	34	69
5. Control—untreated	50	50	38	76
6. Control—untreated	50	49	32	64

TABLE V: INCIDENCE OF GROSSLY MALIGNANT TUMORS (SERIES 2)

Group	Number of mice at start	Effective total	Number grossly malignant	Per cent grossly malignant (Per cent of effective total)
1. Peroxide 1×10^{-3}	50	50	38	76
2. Peroxide 1×10^{-9}	50	49	24	49
3. Peroxide 1×10^{-18}	50	49	26	53
4. Control—distilled water	50	49	24	49
5. Control—untreated	50	50	24	48
6. Control—untreated	50	49	18	36

TABLE VI: INCIDENCE OF VARIOUS TYPES OF MALIGNANT TUMORS IN BENZPYRENE-PAINTED MICE (SERIES 2) *

Group	Effective mouse total	Epidermoid carcinoma: Number of tumors	Epidermoid carcinoma: Per cent	Lung cancer: Number of tumors	Lung cancer: Per cent	Breast cancer: Number of tumors	Breast cancer: Per cent	Other types of tumor	Total, per cent
1. Peroxide 1×10^{-3}	50	45	90	3	6	1	2	.....	98
2. Peroxide 1×10^{-9}	49	30	61	12	24	1	2	1 Fibrosarcoma, 2 per cent	89
3. Peroxide 1×10^{-18}	49	30	61	14	29	2	4	1 Fibrosarcoma, 2 per cent	96
4. Control—distilled H_2O	49	31	63	8	16	0	0	.....	79
5. Control—untreated	50	33	66	9	18	0	0	2 Fibrosarcomas, 4 per cent	88
6. Control—untreated	49	27	55	10	20	0	0	3 Fibrosarcomas, 6 per cent	81

* Percentages are calculated on basis of effective total mice for each group. Diagnosis based on microscopic examination.

shown in Figs. 8 and 9. It can be seen that in general the curves in all the experimental and control groups have the same shape and slope and hence the tumors grew at about the same rate, within the experimental error. Rapidly decreasing numbers of animals after about 90 days are probably responsible for the greater discrepancy between the curves in the last stages.

DISCUSSION

On inspection of the curves and tables which show the incidence of papillomas and malignant tumors and their rate of growth, it is evident that in series 1 there is no correlation between peroxide administration and tumor production as reported by Maisin. The control group occupies an intermediate position while the peroxide groups fall on either side of it, a result compatible with ordinary conditions of chance or variability.

In series 2, precisely the same may be said except for the incidence of epidermoid carcinoma in group I which received peroxide in concentration of 1×10^{-3}, a "stimulating" dose. Whether the larger number of epidermoid carcinomas in this group is actually due to "stimulation" is, however, open to question and hinges primarily upon the normal maximum variation that can be expected when reasonably large numbers of mice are treated cutaneously with benzpyrene. In series 1 the difference between the highest (78 per cent) and lowest (55 per cent) incidence of epidermoid carcinoma is 23 per cent. In series 2 the difference between the highest incidence (90 per cent) and the lowest (55 per cent) is 35 per cent. Since no prophylactic effect is evident from those peroxide concentrations which should give protection against benzpyrene, it seems more likely that the extremes encountered in each of these two series of benzpyrene painting experiments are the maximum and minimum of normally variable yields of these skin cancers in this strain under these experimental conditions.

It is of interest to note that among eight groups of controls cited by Maisin in various experiments, the highest yield of malignant tumors was 69 per cent and the lowest 18 per cent, a difference of 51 per cent. It would be instructive to see whether under his experimental conditions he would have secured a range of similar magnitude had he used 3 or 4 control groups at the same time in any one experiment instead of one control group for all experimental groups.

SUMMARY

In two series of experiments mice of the Bagg Albino strain were painted cutaneously with benzpyrene, 400 in the first series, 300 in the second series. Some of the mice received biweekly injections of aqueous solutions of di-(hydroxymethyl) peroxide in various concentrations, some received distilled water, and others acted as untreated controls. From the standpoint of tumor incidence and rate of tumor growth, no effect of peroxide administration was evident, either stimulating or inhibitory.

III. Tumors Induced by Subcutaneous Injection of Benzpyrene

Eight hundred mice of the Bagg albino strain were injected subcutaneously with benzpyrene. They were 8 to 12 weeks old at the start of experiment, and were divided proportionately among eight groups as regards age and sex. The mice were raised in this laboratory and were the progeny of mice originally secured from the Roscoe B. Jackson Memorial Laboratory. Diet and housing conditions were the same as for previous experiments, as was the preparation of the di-(hydroxymethyl) peroxide used for injection (1).

Each mouse was injected subcutaneously in the right flank with 10 mgm. of crystalline benzpyrene (obtained from Meurice, Brussels), dispersed in C.P. glycerine. The mice were then divided into eight groups and treated as follows: group 1, peroxide 1×10^{-3}; group 2, peroxide 1×10^{-9}; group 3, peroxide 1×10^{-18}; group 4, controls, distilled water; groups 5, 6, 7, 8, controls, untreated.

Each of the first four groups received every 2 weeks 0.5 cc. of an aqueous solution of their respective peroxide concentrations, or of distilled water. The remaining four groups served as untreated controls. The carcinogen was injected on September 13, 1938; peroxide injections were begun the following day, and were continued till the death of each animal.

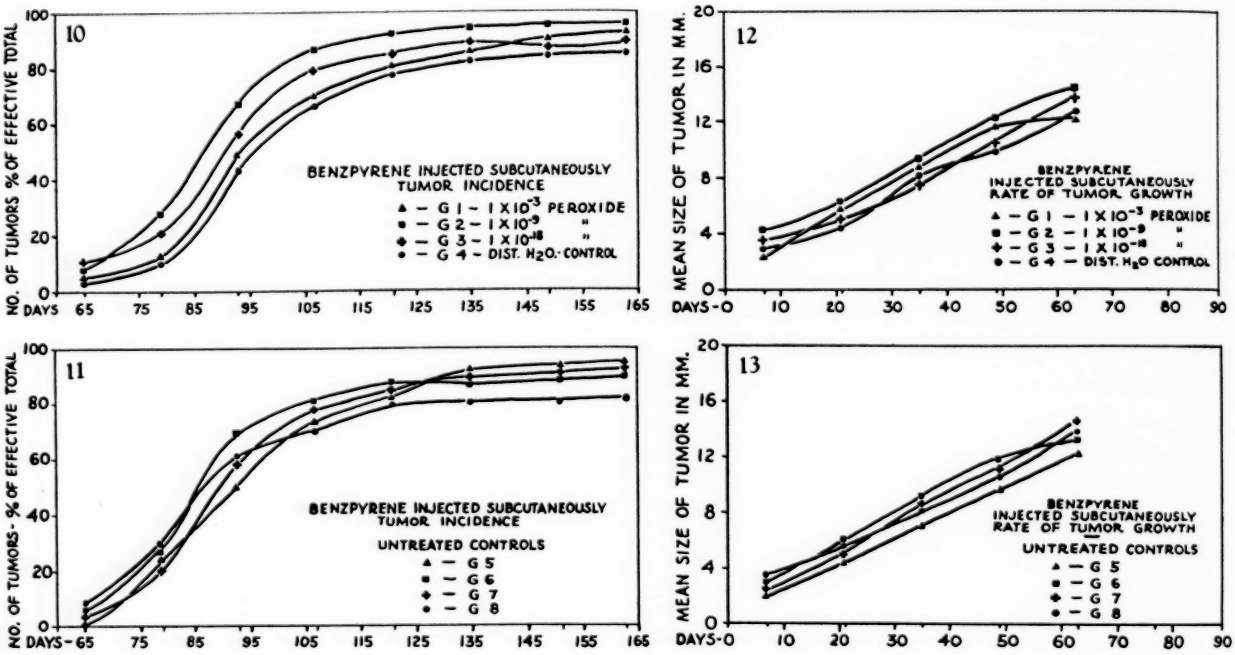
Beginning one month after injection of the carcinogen, examinations were performed every 2 weeks for onset of tumor. Although initial thickening at the site of injection was noted as such, a growth was arbitrarily not considered a tumor unless the average of its three diameters was 5 mm. or greater. Table VII shows the number of tumors secured in each group.

It can be seen from the figures in Table VII that there is no evidence of any peroxide effect as reported by Maisin and his collaborators insofar as the final number of tumors which arose in each group is concerned.

The rate at which the tumors appeared in the experimental and control groups is shown in Figs. 10 and 11. Inspection of these curves shows excellent

TABLE VII: NUMBER OF TUMORS PRODUCED BY BENZPYRENE INJECTED SUBCUTANEOUSLY

Group	Number of mice at start	Effective total	Number of tumors	Per cent of tumors (Per cent of effective total)
1. Peroxide 1×10^{-3}	100	91	85	93
2. Peroxide 1×10^{-9}	100	90	87	97
3. Peroxide 1×10^{-18}	100	91	82	90
4. Control—distilled water	100	92	82	89
5. Control—untreated	100	98	91	93
6. Control—untreated	100	96	89	93
7. Control—untreated	100	96	88	92
8. Control—untreated	100	95	86	91



FIGS. 10 TO 13

TABLE VIII: PER CENT DISTRIBUTION OF VARIOUS TYPES OF TUMORS INDUCED BY BENZPYRENE INJECTED SUBCUTANEOUSLY

Group	Epidermoid carcinoma, per cent	Fibrosarcoma, per cent	Rhabdomyosarcoma, per cent	Lung cancer, per cent
1. Peroxide 1×10^{-3}	35	70	42	3
2. Peroxide 1×10^{-9} *	33	72	37	4
3. Peroxide 1×10^{-18} *	34	67	40	1
4. Control—distilled water	25	71	42	1
5. Control—untreated	28	81	41	1
6. Control—untreated	38	68	47	1
7. Control—untreated	26	71	44	2
8. Control—untreated	42	81	34	3

* In group 2, one case of melanotic sarcoma, and in group 3 one case of adenocarcinoma of the pancreas were also found.

agreement of tumor incidence between the experimental and control mice.

A graphic comparison between the rate of growth of the tumors among the various groups in this experiment is given in Figs. 12 and 13. Again there is good agreement of growth rate between experimental and control mice, especially during the first 10 to 12 weeks. Beyond that period rapidly decreasing numbers result in a somewhat greater discrepancy.

Histologic examination of the subcutaneous tumors showed that they consisted of epidermoid carcinomas, fibrosarcomas, and rhabdomyosarcomas. Each tumor type appeared singly and in combination with one or both of the other two types. There was also one case of melanotic sarcoma (group 2) and one case of adenocarcinoma of the pancreas (group 3). A very small number of lung cancers were found in each group. The Bagg albino mouse used in this experiment is reported to have an incidence of spontaneous lung cancer of about 20 per cent; but the rapid onset of tumor and death of the animal probably precluded the survival of the host to an age when lung cancer ordinarily appears.

Treating each type of neoplasm as a separate entity, their percentage distribution among the various groups is given in Table VIII. Inspection of these data shows no significant difference in number and distribution of any one of the different types of tumor encountered, beyond what might be expected as the result of random contact of the carcinogen with the subcutaneous tissues.

SUMMARY

Eight hundred mice of the Bagg albino strain, divided into eight groups of 100 mice each, were injected subcutaneously with benzpyrene. Some groups received bimonthly injections of di-(hydroxymethyl) peroxide, one group received distilled water, four groups served as untreated controls.

From the standpoint of total number and type of tumor produced in each group, rate of tumor incidence, and rate of tumor growth, there is no evidence that under these experimental conditions peroxide administration exerted either a stimulating or a preventive effect.

It is a pleasure to acknowledge the technical assistance of Miss Janice C. Humphrey and Mr. Bernard S. Meyrowitz.—Author.

REFERENCES

1. Belkin, M. The Lack of Influence of Di-(Hydroxymethyl) Peroxide on the Incidence and Growth of Transplanted, Induced, and Spontaneous Mouse Tumors. I. Transplanted Tumors. Cancer Research, **2**:264-268. 1942.

The Lack of Influence of Di-(Hydroxymethyl) Peroxide on the Incidence and Growth of Transplanted, Induced, and Spontaneous Mouse Tumors*

IV. Spontaneous Tumors in the Dba and C3H Strains

Morris Belkin, Ph.D.

(From the Department of Pathology, Yale University School of Medicine, New Haven, Conn.)

(Received for publication January 7, 1942)

In previous communications (1, 2), experiments were described in which di-(hydroxymethyl) peroxide was injected into mice painted with benzpyrene. Although Maisin and his collaborators [1] have stated that administration of this substance resulted in a diminished incidence of cancer in mice treated with this carcinogen, no evidence for this contention was found in the experiments reported previously. This was true not only for mice painted with benzpyrene, but for mice in which benzpyrene was injected subcutaneously, as well as for animals which carried a transplanted breast tumor.

The present report deals with experiments in which the administration of di-(hydroxymethyl) peroxide was extended to include mice bearing tumors of spontaneous origin.

The diet and housing conditions of the mice and the preparation and use of the di-(hydroxymethyl) peroxide [2] were the same as described in the first paper of this series (1).

SPONTANEOUS BREAST TUMORS IN DILUTE BROWN MICE

Two hundred and ninety-seven mice of the dilute brown (Dba) strain were received in six lots from the Roscoe B. Jackson Memorial Laboratory over a period of 6 weeks beginning April 6, 1938. Each lot was proportionately distributed among three groups. Each group of mice received the following treatment: group 2, 100 mice, peroxide 1×10^{-10}; group 3, 100 mice, peroxide 1×10^{-19}; group 4, 97 mice, controls, untreated. Each animal received every 2 weeks 0.5 cc. of an aqueous solution of its respective peroxide concentration subcutaneously and at a distance from any tumor present. Also, each animal was mated and permitted to have and nurse one litter to weaning age (25 days). This experiment was set up by Dr. J. Maisin who also participated in making the first few peroxide injections.

Examination for tumors was made every 2 weeks. Arbitrarily, a growth was not considered a tumor unless it was 5 mm. in diameter or larger. The experiment was terminated after 26 months, the last surviving mice being sacrificed at that time.

TABLE I: INCIDENCE OF SPONTANEOUS BREAST TUMORS IN DBA STRAIN

Group	Number of mice at start	Effective total	Number of tumors	Per cent tumors (Per cent of effective total)
2. Peroxide 1×10^{-10}	100	93	27	29
3. Peroxide 1×10^{-19}	100	68	28	41
4. Control—untreated	97	84	34	40

The total number of palpable spontaneous tumors which appeared in each group is given in Table I.

The rate at which these tumors arose from the standpoint of the age of the mice is shown in Fig. 1. In this figure the ordinate is the effective total mice for each group and the abscissa their age. Inspection of the curves indicates that there was no significant difference in tumor incidence, especially during the first 500 days or so.

The tumors were measured along their three diameters with calipers, and their mean expressed in mm. was used as an index of tumor size. The error is about ± 15 per cent.

The rate at which these tumors grew is indicated in Fig. 2. There is greater discrepancy in these growth curves than has been obtained in other experiments with transplanted tumors and tumors induced with

* This investigation was aided by a grant from The Hubbard-McCormick Clinical Cancer Research Fund.

[1] For references to work of Dr. Maisin and collaborators see (1).

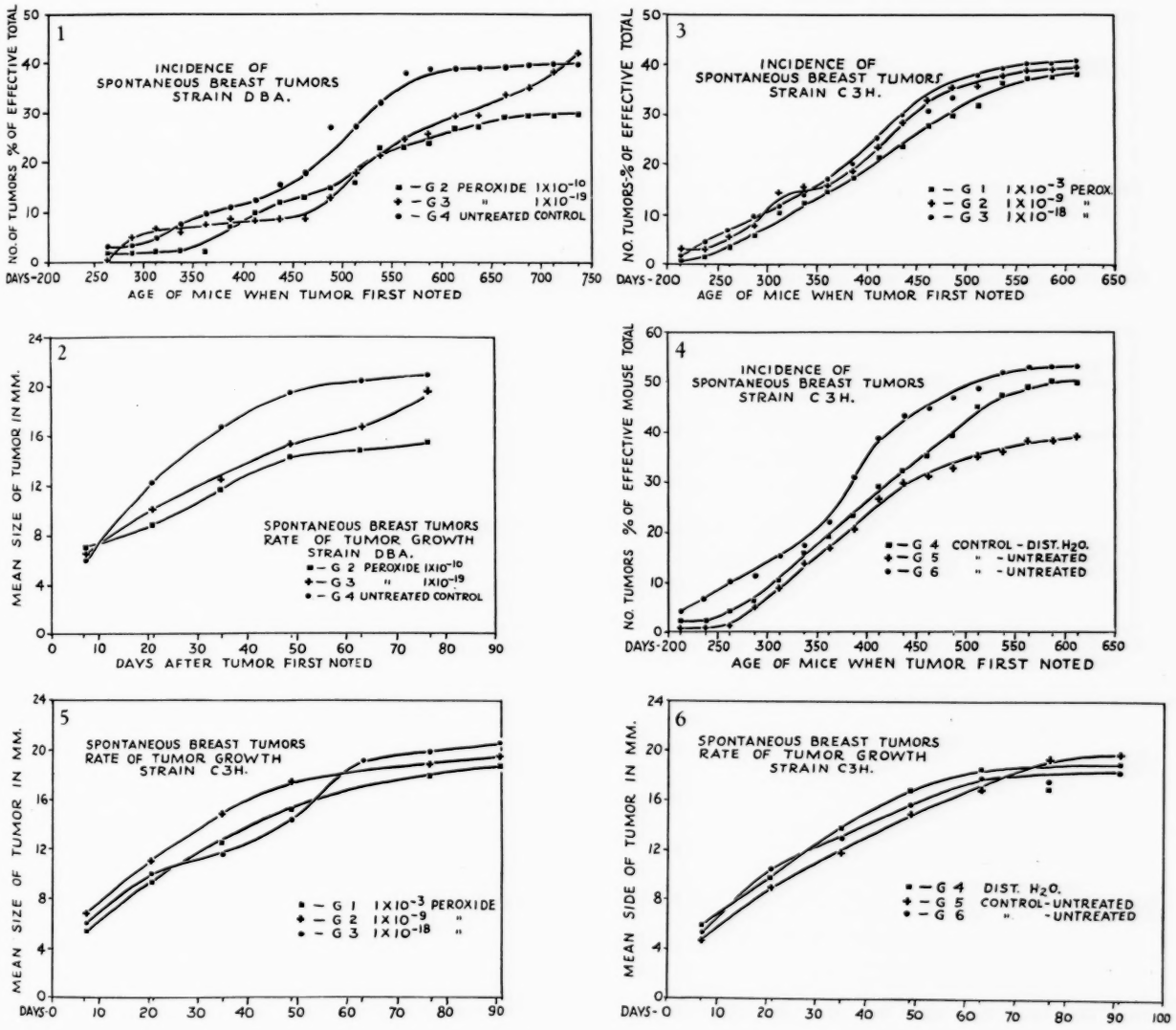
[2] Hereafter referred to as peroxide.

benzpyrene (1, 2), which in all probability is due to the relative paucity of data.

Histologic examination of the breast tumors revealed them as adenocarcinomas of various kinds. A few tumors considered grossly as spontaneous breast tumors and which arose in sites typical for breast tumors proved to be of different tissue origin. The

DISCUSSION

The highest tumor incidence of spontaneous breast tumors (40 per cent) arising in any group in this experiment is still considerably lower than the 65 to 100 per cent incidence reported by other investigators for this strain. No immediate explanation for this phenomenon is at hand, but in this connection it may



FIGS. 1 TO 6

number and different types of tumors arising in each group, as diagnosed by microscopic examination, are given in Table II. Since it is held that the preventive action of peroxide should be the same for all tumors, regardless of tissue origin, all the various tumor types encountered are given, although attention was primarily centered on spontaneous breast tumors.

It can be seen that there is no apparent prophylactic action of the peroxide in either of the groups receiving this substance as compared with the control group.

perhaps be pertinent to note that at the beginning of the experiment a paratyphoid epidemic arose in these mice. Since it has been shown by other investigators that certain bacterial products have the power of producing hemorrhage in and otherwise attacking transplanted and induced tumors, the interesting possibility arises as to what effect a previous infection may have on the subsequent spontaneous tumor history of mice exposed to bacterial products in that manner.

SUMMARY

Two hundred ninety-seven mice of the dilute brown (Dba) strain were divided into three groups. The first two groups received every 2 weeks aqueous injections of di-(hydroxymethyl) peroxide; the third group served as untreated controls. All the mice were mated once and then examined periodically for spontaneous tumor.

From the standpoint of total number and type of tumor arising in each group, rate of tumor incidence, and rate of tumor growth, there is no evidence that under these experimental conditions peroxide administration exerted either a stimulating or preventive effect.

SPONTANEOUS BREAST TUMORS IN STRAIN C3H

Ninety C3H female mice, 4 to 6 weeks old, obtained from the Roscoe B. Jackson Memorial Laboratory, were divided into six groups of 15 each. When the mice were 8 weeks old they were permitted to litter and nurse their young to weaning age (25 days). The mothers were then mated again and this time the young were removed at birth. Female progeny of the first litters were added proportionately to each group until a total of 100 mice per group was obtained. These were also bred twice as described above. This experiment was begun February 14, 1939, and was terminated after 25 months. Conditions of diet, housing, and preparation of di-(hydroxymethyl) peroxide were the same as for the previous experiments (1).

The mice in these six groups received the following treatment: group 1, peroxide 1×10^{-3}; group 2, peroxide 1×10^{-9}; group 3, peroxide 1×10^{-18}; group 4, distilled water, controls; groups 5, 6, untreated controls.

The first four groups received subcutaneously every 2 weeks 0.5 cc. of an aqueous solution of their respective peroxide concentrations or of distilled water. These injections were always at a distance from any tumor present.

Peroxide injections were begun when the female was mated and continued till the death of the animal. Examinations for tumors were made every 2 weeks. The tumors were measured along their three diameters and, as before, the mean of these measurements, expressed in mm., was used as an index of tumor size. As in the case of the experiment with the Dba strain reported previously, a growth was not considered a tumor unless it was 5 mm. in diameter or larger. The total number of palpable spontaneous tumors which appeared in each group is given in Table III.

It can be readily seen that on the basis of gross appearance of tumors the peroxide-treated groups are

TABLE II: TOTAL INCIDENCE OF SPONTANEOUS TUMORS IN STRAIN DBA *

Group	Spontaneous breast cancer: Number of tumors	Spontaneous breast cancer: Per cent	Lymphosarcoma: Number of tumors	Lymphosarcoma: Per cent	Other types of tumor	Other types of tumor: Number of tumors	Other types of tumor: Per cent	Total: Number of tumors	Total: Per cent
2. Peroxide 1×10^{-10}	26	28	23	25	1 Lung adenocarcinoma	1		53	57
					1 Lung squamous cell carcinoma	1			
					2 Fibrosarcoma	2			
3. Peroxide 1×10^{-19}	24	35	17	25	3 Lung adenocarcinoma	4		48	70
					3 Fibrosarcoma	4			
					1 Epidermoid carcinoma	2			
4. Control—untreated	34	40	14	17	1 Fibrosarcoma	1		50	59
					1 Rhabdomyosarcoma	1			

* All percentages are calculated on the basis of the effective total mice for each group. Diagnosis based on microscopic examination.

TABLE III: INCIDENCE OF SPONTANEOUS BREAST TUMORS IN C3H MICE

Group	Number of mice at start	Effective total	Number of tumors	Per cent tumors (Per cent of effective total)
1. Peroxide 1×10^{-3}	100	100	39	39
2. Peroxide 1×10^{-9}	100	100	39	39
3. Peroxide 1×10^{-18}	100	100	40	40
4. Control—distilled water	100	100	50	50
5. Control—untreated	100	100	39	39
6. Control—untreated	100	100	53	53

identical with regard to tumor incidence, and no effect, either stimulating or preventive, is discernible. In two of the control groups tumor incidence is about 10 per cent (group 4) and 13 per cent (group 6) higher than in any of the first three groups. This is considered as normal variability under these experimental conditions. Group 5, an untreated control group, is identical with the peroxide groups.

The rate at which these tumors arose in the various groups is shown in Figs. 3 and 4. The ordinates in these curves is per cent of the effective total mice for each group, and the abscissae the age of the mice when the tumor first appeared. Inspection of these curves discloses the fact that no effect of peroxide was evident at any time.

C3H strain. The differences among the groups are within the normal range of biological variability.

SUMMARY

Six hundred C3H mice were divided into six groups of 100 mice each. The first 3 groups received subcutaneous injections of an aqueous solution of di-(hydroxymethyl) peroxide in a different concentration for each group. One group acted as a distilled water control, two groups as untreated controls. All the mice were mated twice, the second litter being removed at birth.

Insofar as total number and type of tumor arising in each group, rate of tumor incidence, and rate of tumor growth are concerned, there is no evidence that under the conditions of these experiments peroxide administration had either a stimulating or inhibiting effect.

TABLE IV: TOTAL INCIDENCE OF SPONTANEOUS TUMORS IN C3H MICE *

Group	Spontaneous breast cancer: Number of tumors	Spontaneous breast cancer: Per cent	Other types of tumor	Other types of tumor: Number of tumors	Other types of tumor: Per cent	Total: Number of tumors	Total: Per cent
1. Peroxide 1×10^{-3}	38	38	1 Fibrosarcoma	1		42	42
			1 Melanosarcoma	1			
			1 Lung adenocarcinoma	1			
			1 Rhabdomyosarcoma	1			
2. Peroxide 1×10^{-9}	40	40	2 Lung adenocarcinoma	2		46	46
			4 Lymphosarcoma	4			
3. Peroxide 1×10^{-18}	43	43	2 Lymphosarcoma	2		45	45
4. Controls—distilled water	50	50	1 Epidermoid carcinoma	1		52	52
			1 Lung adenocarcinoma	1			
5. Controls—untreated	38	38	1 Lymphosarcoma	1		39	39
6. Controls—untreated	53	53	1 Fibrosarcoma	1		55	55
			1 Lymphosarcoma	1			

* Percentages are based on the effective total mice for each group. Diagnosis made by microscopic examination.

In Figs. 5 and 6 where the rate of tumor growth is shown, it is evident that there is good agreement (within the experimental error which is ±15 per cent) between the growth rate of the tumors in all the groups, whether they received peroxide or not.

As in the case of the dilute brown strain, histologic examination of the tumors showed that a few tumors grossly considered of breast origin were of other types. Also, a few breast tumors, found at autopsy as breast nodules, and shown microscopically to be cancer, were found. The final figures as to breast cancer found in each group are given in Table IV. These were all adenocarcinomas of various kinds. As before, other types of tumors are included.

Examination of the data in this table once again shows the lack of effect of peroxide administration with regard to spontaneous tumor incidence in the

It is a pleasure to acknowledge the technical assistance of Miss Janice C. Humphrey and Mr. Bernard S. Meyrowitz.—Author.

REFERENCES

1. BELKIN, M. The Lack of Influence of Di-(Hydroxymethyl) Peroxide on the Incidence and Growth of Transplanted, Induced, and Spontaneous Tumors. I. Transplanted Tumors. Cancer Research, **2**:264-268. 1942.
2. BELKIN, M. The Lack of Influence of Di-(Hydroxymethyl) Peroxide on the Incidence and Growth of Transplanted, Induced, and Spontaneous Tumors. II. Tumors Induced by Cutaneous Painting with Benzpyrene. III. Tumors Induced by Subcutaneous Injection of Benzpyrene. Cancer Research, **2**:269-275. 1942.

The Effect of Maternal Influence upon Spontaneous Leukemia of Mice*

J. Furth, M.D., R. K. Cole, Ph.D., and M. C. Boon, M.A.

(From the Department of Pathology, Cornell University Medical College and New York Hospital, New York, N. Y.)

(Received for publication January 14, 1942)

The effect of maternal influence upon spontaneous leukemia was first investigated by MacDowell and Richter (6). Reciprocal crosses between mice of their high (H) and low (L) leukemia stock gave the following values for spontaneous leukemia:

H/L[1]	61% leukemia	(139 mice)
L/H	42% leukemia	(106 mice)

They state that reciprocal fostering did not influence the incidence of leukemia but no figures are given.

In a similar study made in this laboratory (4) with two different stocks, reciprocal crosses yielded the following values for the incidence of spontaneous leukemia:

H/L	21.9% leukemia	(192 mice)	28.1% in ♂ 15.6% in ♀
L/H	11.6% leukemia	(173 mice)	8.8% in ♂ 14.6% in ♀

In contrast to the observations of MacDowell and Richter, fostering in experiments made in this laboratory lowered the incidence of leukemia in the high leukemia stock but did not produce leukemia in the low leukemia stock (1) as shown by the following figures:

H not fostered	60% leukemia	(432 mice)
H fostered by L	32% leukemia	(176 mice)
L not fostered	2% leukemia	(510 mice)
L fostered by H	2% leukemia	(181 mice)

These figures suggested the possibility of some maternal influence and for this reason a new series of experiments was undertaken: (a) Mice of the high leukemia stock (Ak) were crossed with mice of a low leukemia stock (C3H) which carries a strong milk influence for mammary carcinoma, thus enabling quantitative observations in the same experiments on manifestations of a known milk influence (Table I). (b) For the same purpose reciprocal fosterings were made between Ak and C3H mice (Table II).

Table I shows that the incidence of leukemia was somewhat lower when the C3H stock was used as the dam. This effect was observed for both the male and female progeny. The progeny from the C3H dam show effect of the milk factor as indicated by a much higher incidence of breast cancer. Thus all three sets of data available (4, 6, and those above) give significantly higher values for leukemia in H/L crosses than in L/H crosses.

This effect is not due to differences in age at death as indicated by comparison of Figs. 1 and 2. When the high leukemia stock was used as a dam, the peak in the incidence of leukemia occurred at 8 to 10 months, in the reciprocal cross at 12 months. A relatively large proportion of hybrid mice lived beyond 16 months of age and had sufficient opportunity to demonstrate a genetic tendency for the development of leukemia.

TABLE I: THE INCIDENCE OF LEUKEMIA AND TUMORS IN RECIPROCAL CROSSES BETWEEN AK AND C3H MICE

Mice			Incidence of neoplasms, per cent		
Stock		Number	Breast	Lung	Leukemia
Ak ♀ × C3H ♂	♀ virgin	108	1	2	48
	♂	93	..	3	54
	♀ + ♂ total	201	..	2	50
C3H ♀ × Ak ♂	♀ virgin	111	14	4	39
	♂	94	..	4	28
	♀ + ♂ total	205	..	4	34

The peak of mortality from leukemia occurs at 8 to 9 months in the Ak stock. Thus in both F_1 hybrids, the peak in the leukemic mortality occurs later than in the high leukemia stock (Fig. 3), and the retardation of occurrence of the disease is greater in L/H than in H/L hybrids. The hybrid mice live on an average longer than the Ak mice.

There is a sex difference as regards incidence of leukemia in the reciprocal crosses. In the data from this laboratory the incidences of leukemia among the females of the reciprocal F_1 populations do not significantly differ. On the other hand, there is a highly

* This investigation has been aided by grants from The Lady Tata Memorial Trust, The Jane Coffin Childs Memorial Fund for Medical Research, and The International Cancer Research Foundation.

[1] The first letter designates the dam, the second the sire.

significant difference in the incidence of leukemia between the males of the respective F_1 populations. This observation confirms our previous observations in which it was found that the differences in the incidences of leukemia among the F_1 populations were due entirely to differences in the incidence of the disease among the male populations, the female populations showing no significant difference.

The milk factor of the C3H stock dam finds expression in the development of breast cancer in 14 per cent of virgin C3H/Ak females as compared to 1 per cent in the reciprocal crosses. It is noteworthy that in our series of virgin C3H females the incidence of breast cancer was almost the same as in the C3H/Ak hybrids (15 per cent in 79 animals).

Table II shows that fostering C3H mice by Ak stock dams lowers the incidence of breast cancers. The relatively high per cent of breast cancer among the fostered mice may be a consequence of the technic. The exchange of suckling mice was made only twice a day and a certain number of mice must have received a small quantity of milk from their natural mothers. Experiments of Bittner (2) indicate that the quantity of milk necessary to induce breast cancer is minute.

TABLE II: EFFECT OF RECIPROCAL FOSTER NURSING ON THE INCIDENCE OF LEUKEMIA AND TUMORS IN STOCK AK AND C3H

Stock	Mice	Number	Incidence of neoplasms, per cent: Breast	Lung	Leukemia
C3H, unfostered:	♀ virgin	79	15	2	1
	♀ bred	112	53	2	0
	♂	107	..	1	0
	♀ + ♂ total	298	..	1.7	0.3
C3H, fostered by Ak:	♀ virgin	79	6	0	0
	♀ bred	24	21	0	0
	♂	112	..	4	0
	♀ + ♂ total	215	..	2	0
Ak, unfostered:	♀ virgin and bred	117 †	2 *	0	61
	♂	100	..	1	53 ‡
	♀ + ♂ total	217	..	0.5	58
Ak, fostered by C3H:	♀ virgin	90	2	0	33
	♀ bred	23	17	0	26
	♂	108	..	1	22
	♀ + ♂ total	221	..	0.5	27
Ak, parents fostered by C3H	♀ virgin	117	7	0	64
	♀ bred	15	13	0	53
	♂	84	..	0	50
	♀ + ♂ total	216	..	0	59

* These breast tumors occurred in virgin females. Previously the incidence of breast tumor in Ak mice was less than 1 in 1,000.
† Of the 117 mice, 89 were bred.
‡ Two cases of splenic tumor in mice of stock Ak have been included among the leukemias.

As regards leukemia the table shows that nursing by Ak mice did not render C3H mice leukemic, as already noted by MacDowell and Richter (6) and Barnes and Cole (1).

On the contrary, fostering Ak mice by C3H dams lowers significantly the incidence of leukemia in both sexes. This confirms the previous observations of Barnes and Cole, using a different low leukemia stock and the same high leukemia stock.

This effect, unlike the Bittner factor, is not transmitted to the progeny for, as Table II shows, offspring of fostered Ak mice have as high an incidence of leukemia as nonfostered Ak stock—but the milk factor for breast cancer persists also in our experiments.

These changes in the incidence of leukemia are not due to differences in longevity, as indicated by comparison of Figs. 5 and 7, and Figs. 6 and 8. With respect to the female populations the progeny of the fostered Ak mice showed the typical curve for mortality from leukemia, as well as from other causes. The fostered mice showed a much lower incidence of leukemia and a marked tendency for a majority of the mice to live well beyond the usual peak of mortality from leukemia. A similar trend was noted for the two male populations, as shown in Figs. 6 to 8.

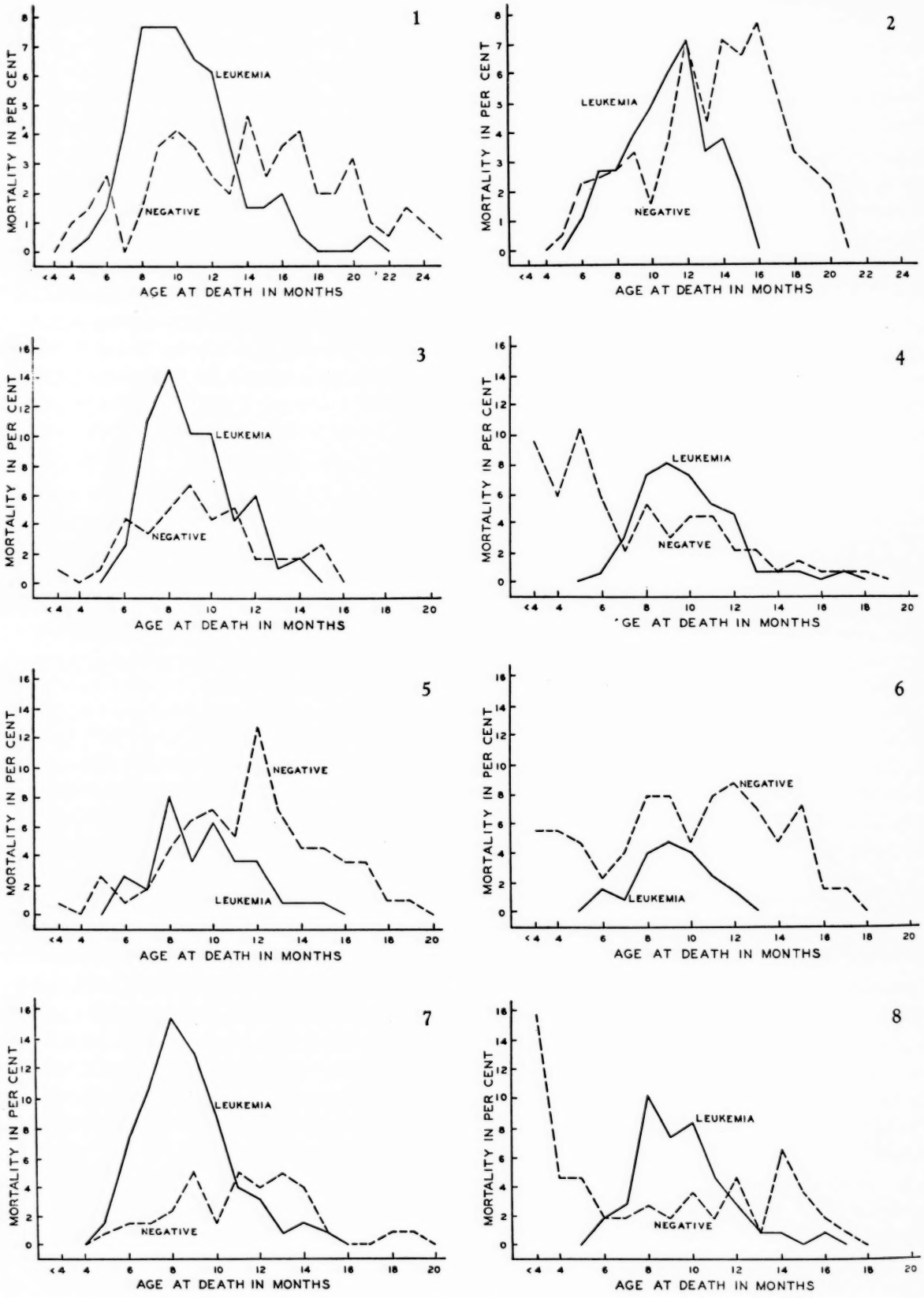
The incidence of lung tumor in the stock studied was low and the data are not sufficient for analysis.

DISCUSSION

The data from three sources, MacDowell and Richter (6), Cole and Furth (4), and those here given, indicate the existence of a maternal influence demonstrable by reciprocal crosses; our data also show this effect by foster nursing (Barnes and Cole and current experiments), while MacDowell and Richter found no evidence of a nursing influence. Unlike the milk factor producing breast cancer, described by Bittner (2, 3), the maternal influence for leukemia is not transmitted to the next generation.

This maternal influence is strong in male mice but slight in female mice. Differences in longevity do not account for this difference in incidence of leukemia as shown by comparison of the corresponding figures.

These observations can be explained in two different ways: 1. The maternal influence is similar to that described by Bittner for breast carcinoma but it is not strong enough to render the mice of the two different stocks tested (Rf and C3H) leukemic. Diminution of this leukemia influence by foster nursing will, however, lower the incidence of leukemia in susceptible mice. Some stocks may be resistant to milk influence and it will be desirable to foster hybrids with low incidence of leukemia but high percentage of Ak inheritance by Ak mice. These hybrids are expected to show a greater susceptibility to the hypothetical


1
MORTALITY IN PER CENT
LEUKEMIA
NEGATIVE
AGE AT DEATH IN MONTHS
2
MORTALITY IN PER CENT
LEUKEMIA
NEGATIVE
AGE AT DEATH IN MONTHS
3
MORTALITY IN PER CENT
LEUKEMIA
NEGATIVE
AGE AT DEATH IN MONTHS
4
MORTALITY IN PER CENT
LEUKEMIA
NEGATVE
AGE AT DEATH IN MONTHS
5
MORTALITY IN PER CENT
NEGATIVE
LEUKEMIA
AGE AT DEATH IN MONTHS
6
MORTALITY IN PER CENT
NEGATIVE
LEUKEMIA
AGE AT DEATH IN MONTHS
7
MORTALITY IN PER CENT
LEUKEMIA
NEGATIVE
AGE AT DEATH IN MONTHS
8
MORTALITY IN PER CENT
LEUKEMIA
NEGATIVE
AGE AT DEATH IN MONTHS

FIGS. 1-8

milk factor of Ak stock than pure C3H mice. The absence of the maternal influence in the second generation suggests that this influence is different from that of the breast cancer influence.

2. It is possible that there exists an inhibitory factor in the low leukemia stock, transferable by foster nursing. The hybridization of the high leukemia stock Ak to two different low leukemia stocks (Rf and C3H) indicates a factor of resistance greater in Rf than in C3H mice as indicated by the following figures:

	Pure stocks			F_1 hybrids			
	Ak	Rf	C3H	Ak/Rf	Rf/Ak	Ak/C3H	C3H/Ak
Per cent leukemia	70	2	1	21.9	11.6	54	36

The fostering experiments do not show this difference, the Rf foster dams reducing the incidence of leukemia in Ak stock mice to 32 per cent, the C3H dams to 33 per cent.

The difference in the behavior of the sexes, one showing a high susceptibility and the other a low susceptibility to the milk factor, requires further elucidation. One observation may be of significance; namely, that in the first few months of adult life mortality is high among the male mice as a result of fighting. It is possible, though not probable, that this may eliminate a larger portion of mice in the preleukemic state.

Transmission experiments of leukemias into these three different stocks of mice (5, 7, and unpublished data) indicate a resistance factor in Rf mice which is almost absent in C3H mice. Spontaneous leukemia arising in Ak mice can be grafted to a high percentage of C3H mice but not at all to Rf mice. The mechanism of tumor transmission is different from that of spontaneous development of neoplasms yet in this instance a relation exists which deserves further study.

Differences in intensity of the milk influence are indicated by foster nursing of low breast cancer mice, Ak, to high breast cancer mice, Af and C3H. The incidence of breast cancer in Af and C3H mice was approximately the same, but the former failed to render Ak mice cancerous while the latter did.

SUMMARY

Reciprocal crosses and reciprocal foster nursings were made between a high leukemia stock, Ak, and a low leukemia, high breast cancer stock, C3H. The incidence of leukemia in the C3H/Ak hybrids was significantly lower than in the reciprocal F_1 generation and the difference was greater between the males.

Foster nursing by low leukemia dams significantly lowered the incidence of leukemia in the high leukemia stock but the next generation behaved as nonfostered mice. Thus this nursing influence is not transmitted to the offspring.

The reciprocal nursing failed to be productive of leukemia in the C3H mice.

Leukemia tends to occur at a later age in F_1 hybrids than in the pure high leukemia stock mice.

REFERENCES

1. Barnes, W. A., and R. K. Cole. The Effect of Nursing on the Incidence of Spontaneous Leukemia and Tumors in Mice. Cancer Research, **1**:99-101. 1941.
2. Bittner, J. J. Breast Cancer and the Pedigree Relationship of Fostered A Stock Mice. Pub. Health Rep., **54**:1642-1650. 1939.
3. Bittner, J. J. Changes in the Incidence of Mammary Carcinoma in Mice of A Stock. Cancer Research, **1**:113-114. 1941.
4. Cole, R. K., and J. Furth. Experimental Studies on the Genetics of Spontaneous Leukemia in Mice. Cancer Research, **1**:957-965. 1941.
5. Furth, J., and W. A. Barnes. Differences Between Malignant Blood Cells from Induced and Spontaneous Leukemias of Mice. Cancer Research, **1**:17-22. 1941.
6. MacDowell, E. C., and M. N. Richter. Mouse Leukemia; Role of Heredity in Spontaneous Cases. Arch. Path., **20**:709-724. 1935.
7. Schweitzer, M. D., and J. Furth. Susceptibility to Transmitted Leukemia Occurring in Pure Bred and Hybrid Mice. Am. J. Cancer, **37**:224-232. 1939.

DESCRIPTION OF FIGURES 1 TO 8

Graphs showing comparison of mortality from leukemia and from causes other than leukemia. Mortality is given in per cent of the total number of mice included in each group. Age at death is given as the nearest month.

Fig. 1.—Mortality curve in Ak ♀/C3H ♂ mice, including both sexes.

Fig. 2.—Mortality curve in C3H ♀/Ak ♂ mice, including both sexes.

Fig. 3.—Mortality curve in unfostered Ak ♀ mice.

Fig. 4.—Mortality curve in unfostered Ak ♂ mice.

Fig. 5.—Mortality curve in Ak ♀ mice fostered by C3H mice.

Fig. 6.—Mortality curve in Ak ♂ mice fostered by C3H mice.

Fig. 7.—Mortality curve in Ak ♀ mice whose parents were fostered by C3H mice.

Fig. 8.—Mortality curve in Ak ♂ mice whose parents were fostered by C3H mice.

The Association of Blood Cell Factors with the Transplantability of the Brown-Pearce Tumor*

Albert E. Casey, M.D., Louise Pearce, M.D., and Paul D. Rosahn, M.D.†

(From the Laboratories of Pathology and Bacteriology of The Rockefeller Institute for Medical Research, Princeton, N. J., the School of Medicine of St. Louis University, St. Louis, Mo., and the School of Medicine of Louisiana State University, New Orleans, La.)

(Received for publication December 19, 1941)

Previous studies have shown wide variations (1), chiefly related to breed (3), in the reaction of individual rabbits to inoculation with the Brown-Pearce tumor. In some breeds less than 10 per cent of the animals were susceptible, but in others more than 90 per cent became riddled with metastases and died within a very short time after transplantation of the tumor.

It has long been suspected that various blood and tissue cells are concerned in the cellular reactions occurring about neoplastic cells. Alterations in the levels of the hemoglobin and of the various blood cells, for instance, have been observed to correspond with the progress or retrogression of neoplasia and with the cellular reaction about the growths. It has been difficult, however, to obtain proof which would demonstrate whether the cellular response in the blood and the tissue about the tumor foci is the cause of the progress or regression of the artificially transplanted tumor or the metastatic tumor, or is itself the result of the progress or regression (7, 8, 11-20, 22-25, 28).

A new approach is clearly necessary to determine the nature of this relationship, and the present experiments have been designed with this objective. They are based upon definitive studies of normal pretransplantation blood levels in individual animals which have been analyzed in relation to post-transplantation reactions. To state the premise differently, it was thought possible that the transplantability, growth, and spread of the Brown-Pearce tumor might be influenced by factors reflected in the pretransplantation blood formulae. The approach would seem to be entirely reasonable, since pretransplantation levels obviously cannot be influenced by the tumor or its products. It is only recently that the characteristics and hereditary nature of the blood formulae in individual animals has been recognized (2, 5), and previous investigations had therefore not included studies of pretransplantation results, without regard to possible alterations in the blood levels after transplantation of the tumor.

This paper is concerned with the relation of nine blood cell factors (red blood cells, hemoglobin, platelets, total white blood cells, neutrophiles, basophiles, eosinophiles, lymphocytes, and monocytes) to the success of transplantation of the Brown-Pearce tumor.

MATERIAL AND METHODS

The study was begun with 195 young adult male rabbits and was concluded with 108 animals selected on the basis of rigid criteria to be described later. Seventeen sets of experiments were carried out on these animals between the years 1927 and 1937.

The animals originally selected were mature and apparently healthy, as evidenced by body weight and the size of the testes, as well as by the absence of canker, snuffles, mange, and diarrhea. They ranged in age from 5 to 12 months. Sixty-one were from standard breeds, as follows: Havana 14, Himalayan 12, English 9, Dutch 5, Belgian and Chinchilla 4 each, French Silver 3, Flemish Giant, New Zealand Red, and American Blue 2 each, and Sable, Castor Rex, Polish, and Lilac 1 each. Of the 47 hybrid animals, 39 were common brown-grays purchased from dealers (possibly mixtures of New Zealand, Belgian, Flemish, and Chinchilla) and 9 were hybrids reared in the laboratory from the standard breeds listed.

The food of the 35 brown-grays used in the first 8 experiments was limited to cabbage, lettuce, hay, and oats, and the water intake was restricted. The food of the 73 animals used in the remaining 9 experiments consisted of standard rabbit chow containing various vitamins and essential food elements, and free access to food and water was permitted at all times.

Prior to inoculation with the Brown-Pearce tumor a series of blood studies was carried out as follows:

In 2 sets of experiments involving 14 animals the entire examination was carried out the same day. The examination consisted of 4 red blood cell determina-

* This investigation was aided by a grant from The International Cancer Research Foundation.

† Now at Yale University School of Medicine, New Haven, Conn.

tions, 4 hemoglobin and platelet determinations, 5 white blood cell determinations, and 5 differential counts in which a total of 1,000 cells was counted.

In the remaining animals the examinations, which varied from 2 to 12 per animal, were conducted over periods varying from 1 to 4 weeks. Approximately 4 red blood cell and 5 total white blood cell examinations were made on each animal, together with 5 differential counts, in which a total of 1,000 cells was counted. An average of 4 hemoglobin determinations was made on each of 97 animals, and 4 platelet counts were made on each of 72 animals. The technic of the blood examinations has been described elsewhere (6). The repeated hemocytic observations thus secured were averaged for each rabbit, the absolute numbers of the individual white blood cells being used instead of the relative per cent.

Within 3 weeks after the last blood examination had been made, each rabbit received a single inoculation into the right testis of 0.3 cc. of normal saline emulsion of Brown-Pearce tumor tissue. Observations on the progress of the tumor growth were made thereafter at weekly or semiweekly intervals. Animals which had not died in the interim were sacrificed at the end of 60 days; necropsy was performed by a systematic plan, described elsewhere (2), by which the body was divided into 50 areas. Transplantation was said to have been successful if a nodule appeared at the site of inoculation and persisted for 30 days or more, or if tumor tissue were found anywhere in the animal at necropsy.

The individual averages for each blood factor were compared with standard values available from a previous study of repeated and averaged similar determinations in 180 male rabbits of the same age, representing 15 standard breeds, in which such variables as sex, season, time of examination, technical errors, food, housing, and disease were eliminated or held constant (6). The means and standard deviations secured in the earlier study were used in the present study, the normal limits for any single factor level being defined as those points 1.959964 times the standard deviation above or below the standard mean; 95 per cent of normal observations lie between such limits. Because their blood levels were not included within these limits, 79 animals, one or more from each of the 17 sets of experiments, were eliminated from the final analysis. In addition, from the original 195 animals, 7 were discarded because of the development of intercurrent disease, and another was discarded because it was not submitted to necropsy. The 108 animals upon which the reported results are based were therefore selected by a rigid process of elimination.

The success of transplantation was first analyzed by means of the χ^2 test (9, 10, 21), with relation to the number of animals with various pretransplantation blood factor levels above or below the standard means (Table I). The animals were then separated into 2 groups. The first group included those with preinoculation blood factor levels within the intermediate distance above or below the standard mean (standard deviation times 0.67[1]), and the second those with levels beyond the intermediate distance above or below the standard means (0.67 to 1.96 standard deviations above or below the standard mean). Each group was composed of approximately 50 per cent of the determinations. Finally, the means and standard deviations of the preinoculation blood factor levels in the groups in which transplantation was successful were compared with those in which it was not.

RESULTS

In 18 of the 108 animals no primary growth appeared and no tumor tissue was found at necropsy. Transplantation was therefore said to have been unsuccessful. In the remaining 90 animals a nodule appeared at the site of inoculation and persisted for 30 days or more, or tumor tissue was found at necropsy. Transplantation was therefore said to have been successful in these animals.

Analysis of the results of the various blood studies showed that the pretransplantation levels of the blood platelets, neutrophiles, basophiles, eosinophiles, lymphocytes, and monocytes bore no statistically significant relation to the success or failure of transplantation of the Brown-Pearce tumor. Furthermore, the average preinoculation levels for the stated factors were not significantly different in the groups in which transplantation was successful and those in which it failed (Tables I and II).

Transplantation was significantly more unsuccessful among animals with preinoculation red blood cell and hemoglobin levels above the standard mean; among animals with hemoglobin levels within the intermediate distance above and below the mean; and among animals with total white blood cell levels (especially within one standard deviation) below the standard mean. The means and standard deviations of these blood factors were significantly different in the groups in which transplantation was successful and those in which it was not. It was therefore concluded that the red blood cell, white blood cell, and

[1] The standard means and quartile distances employed were, for the various blood cell factors, as follows: 5,370,000, 4,999,000, and 5,741,000 red cells; 69.1, 64.7, and 73.48 per cent hemoglobin; 566,000, 486,000, and 645,600 blood platelets; 7690, 6683, and 8697 white blood cells; 3780, 3038, and 4522 neutrophiles per cu. mm., 500, 331, and 669 basophiles per cu. mm.; 111, 50, and 172 eosinophiles per cu. mm.; 2550, 1855, and 3245 lymphocytes per cu. mm.; 740, 517, and 963 monocytes per cu. mm.

hemoglobin levels were apparently related to the success or failure of transplantation.

DISCUSSION

The red blood cell and hemoglobin levels in individual animals were significantly correlated ($r = +0.666$, $n = 95$, $P = 0.0001$) (10). The relation of the hemoglobin level to the success of transplantation, although similar to that of the red blood cell level, was much greater. No significant correlation, however, existed between the hemoglobin and total white blood cell levels ($r = +0.175$, $n = 95$, not significant). It was concluded, therefore, that the hemoglobin-red cell (as one) and the total white blood cell levels were the only ones of the 9 preinoculation blood cell factors studied which were independently related to the success of intratesticular transplantation of the Brown-Pearce tumor.

In a preliminary publication based on studies of the preinoculation blood factor levels (including 35 animals of the present 108) it was concluded that deviation of certain pretransplantation levels above or below the species mode for a given factor might be related to the subsequent susceptibility of the animals to intratesticular transplantation with the Brown-Pearce tumor (4). Since that report was made it has become evident that the reaction of the rabbit to this tumor should be considered in two phases, namely the success of transplantation, and the later spread of the neoplasm in the body of the host.

The paradoxical relationship of susceptibility to modal deviation apparent in the preliminary study was interpreted by Strong [2] as indicating that many

Table I: Association of Blood Cell Factors with Transplantability of the Brown-Pearce Tumor

Blood factor level		R no.	H no.	P no.	W no.	N no.	B no.	E no.	L no.	M no.
High	S	39	29	24	42	40	50	52	40	38
	U	13	11	3	3	6	8	11	5	8
Low	S	51	55	39	48	50	40	38	50	52
	U	5	2	6	15	12	10	7	13	10
Total		108	97	72	108	108	108	108	108	108
χ^2		5.10	11.660	0.08	5.560	0.75	0.75	0.07	1.71	0.03
P		0.02	—0.001	0.78	0.018	0.40	0.40	0.80	0.20	0.85
Intermediate	S	51	42	30	49	57	43	62	53	52
	U	8	13	4	11	10	12	10	12	13
Extreme	S	39	42	33	41	33	47	28	37	38
	U	10	0	5	7	8	6	8	6	5
Total		108	97	72	108	108	108	108	108	108
χ^2		0.90	11.46	0.00	0.50	0.37	2.14	1.20	0.37	1.32
P		0.38	—0.01	0.99	0.48	0.60	0.15	0.27	0.57	0.25

Note: No. = number of rabbits; R = red blood cells; H = hemoglobin (available on 97 animals only); P = blood platelets (available on 72 animals only); W = white blood cells; N = neutrophiles; B = basophiles; E = eosinophiles; L = lymphocytes; M = monocytes; High = mean + 0.0-1.96 standard deviations; Low = mean − 0.0-1.96 standard deviations; Intermediate = mean ± 0.0-0.67 standard deviations; Extreme = mean ± 0.67-1.96 standard deviations; S = successful transplantation; U = unsuccessful transplantation; χ^2 (see Fisher's *Statistical Methods for Research Workers*); P = probability.

Table II: Preinoculation Blood Factor Levels Among Animals with Successful and Unsuccessful Transplants of the Brown-Pearce Tumor

Transplantation		R	H	P	W	N	B	E	L	M
Successful	n	90	84	63	90	90	90	90	90	90
	Mx	535.26	66.77	537.43	755.33	366.58	55.267	128.78	248.57	71.122
	Sm	5.83	0.713	15	19.0	11.65	2.64	9.4	10.91	3.5
Unsuccessful	n	18	13	9	18	18	18	18	18	18
	Mx	560.11	70.31	548.00	685.33	333.83	49.11	131.67	219.00	67.55
	Sm	101	0.54	39.9	20.92	16.80	4.35	21.82	16.1	8.0
Difference	d	24.85	3.54	10.6	70.00	32.75	6.16	2.89	29.57	3.57
	Sd	± 11.63	± 0.90	± 42.6	± 28.24	± 20.45	± 5.10	± 23.8	± 19.50	± 8.73
	t	2.13	3.9	0.248	2.48	1.6	1.21	0.121	1.516	0.409
	n	106	95	70	106	106	106	106	106	106
	P	0.04	0.001	...	0.013	...	...	...	...	...

Note: R = red cells, H = hemoglobin, P = platelets, W = total white cells, N = neutrophiles, B = basophiles, E = eosinophiles, L = lymphocytes, M = monocytes; n = number of observations; Mx = mean; Sm = standard error of the mean; d = difference between the means; Sd = standard error of the difference; t, n, see Fisher (*Statistical Methods for Research Workers*); P = probability.

[2] Strong's observation that mice of cancer strains developed anemia before the gross demonstration of malignant changes did not settle the problem of which was cause and effect.

of the animals used were diseased, therefore had abnormal blood factor levels, and therefore exhibited increased susceptibility to transplantation (26). The same criticism cannot be made of the present study, since by the rigorous selection practiced, 79 of an original 187 animals, although apparently healthy, were discarded because they presented one or more abnormal blood factor levels. These rejections represented 42 per cent of an apparently healthy population.

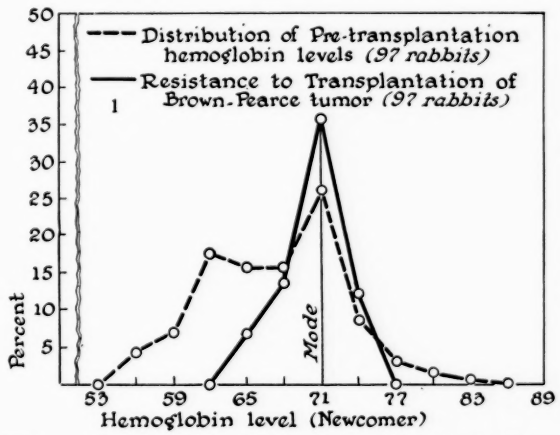
With these variables thus controlled, the possible relationship of marked deviation from the normal as the underlying factor in the success of transplantation of the tumor was investigated in the 108 rigidly selected animals.

1. The distributions of the preinoculation hemoglobin levels (Fig. 1) and total white blood cells (Fig. 2) were plotted in per cent and the modal points noted. The mode for the total white blood cells was 7,000 per cu. mm., and there was a skew to the right. The mode for the hemoglobin was 70.5 per cent, and there was a skew to the left. Both distribution curves were identical within the error of sampling with standard curves for the factors, and the modes were identified with the standard modes (Figs. 1 and 2).

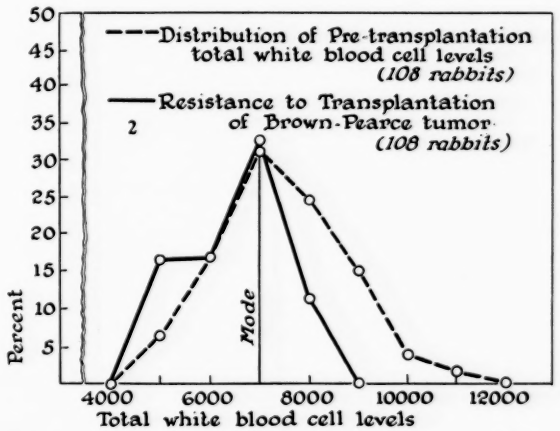
2. The percentage of animals resistant to transplantation was plotted for each preinoculation level and for each factor (Figs. 1 and 2).

The groupings about the mode illustrate graphically the statistically significant relations of the white blood cell and hemoglobin levels to the success of transplantation, the mode and mean of the total blood cell levels being more widely separated from each other than the mode and the mean of the hemoglobin levels. The paradoxical relation of susceptibility to deviations from the mode was more striking in this study than in the preliminary study. In fact, the distribution and the resistance curves were identical except for the skewed portions. Skew values for the hemoglobin-red cell towards anemia and for the white cell values towards leukocytosis were associated with success of transplantation.

When the 79 animals discarded because of abnormal preinoculation blood values were analyzed, no statistically significant relation of the hemoglobin, the red blood cell, and the total white cell levels to the success or failure of transplantation could be demonstrated although the trends were in the same direc-



Resistant	0	0	0	0	1	2	9	1	0	0	0	13
Susceptible	0	4	7	17	14	13	16	7	3	2	1	84
Total	0	4	7	17	15	15	25	8	3	2	1	97



Resistant	1	3	11	3	1				18
Susceptible	1	5	15	23	24	15	4	2	90
Total	1	6	18	34	27	16	4	2	108

FIG. 1.—Paradoxical relation of the distributions in per cent of the hemoglobin level and the transplantability of the Brown-Pearce tumor in normal rabbits. Note the discrepancy for skew values.

FIG. 2.—Paradoxical relation of the distributions in per cent of the total white blood cell level and the transplantability of the Brown-Pearce tumor in normal rabbits. Note the discrepancy for skew values.

tions. The importance of a normal blood formula in experimental work where a standard response is expected is thus demonstrated.

Although the absolute numbers of the various white blood cells were used in the study the levels in relative per cent were calculated for the neutrophiles, basophiles, eosinophiles, lymphocytes, and monocytes. The levels in relative per cent were analyzed through the use of correlation tables, high and low, and intermediate and extreme groupings. No statistically significant relationship of the levels in relative per cent of the success of transplantation of the Brown-Pearce tumor was obtained.

All resistant animals had hemoglobin levels within 0.67 times the standard deviation above or below the mode for normal rabbits, and all but one had total white blood cell levels within this range. The single exception was within the limits of error from the

modal group. Every animal with hemoglobin or white blood cell levels or both above or below these limits was successfully transplanted.

For each character approximately half the observations fell within ± 0.67 times the standard deviation of the mode. Since the total white blood cell and hemoglobin levels are independent variables, it would be expected that one quarter of the 97 animals upon which these values were available would have both levels within this distance of the mode. Actually, 25 animals fell into this group. If resistance to the intratesticular transplantation of the Brown-Pearce tumor were the summation effect of three independent variables with similar frequency distributions and with levels within ± 0.67 standard deviations of the mode, it would be expected that 12.125 animals would be resistant to the neoplasm. Actually, there were 13 resistant animals. These observations strongly suggest that a third unknown variable or variables, together with the hemoglobin-red cell and total white blood cell factors, would fully explain the resistance of the rabbit to intratesticular transplantation of the tumor by the methods employed.

A search among other blood factors studied and the construction of correlation tables of various sorts failed to reveal any possible linear or alinear relation other than that of the hemoglobin-red cell and total white blood cells. The small number of observations on the blood platelets unfortunately did not permit adequate analysis. No relationship of the red blood cell level to the success of transplantation could be demonstrated when the hemoglobin and total white blood cell levels were held constant. The total white blood cell value was found to be the summation effect of each of the constituent white blood cell elements (which were insignificant in themselves) and not of one or two elements individually. This observation suggests that factors affecting blood volume were responsible for the relationship of the total white blood cells to the success of transplantation rather than the white blood cells *per se*. Variations in the plasma protein levels is a possibility (27).

The distance mode ± 0.67 standard deviations was selected arbitrarily and was employed only because it divided the data into two approximately equal parts; it would be quite possible to employ some other division. The fact that quantitative data such as blood cell levels can be altered by environment as well as by inheritance suggests a basis for the study of the relative effects of heredity and environment. The importance of blood factor level variations such as these emphasizes the danger of limiting observations to a single inbred strain of animals or to too few standard strains in the study of factors involved in mammalian resistance.

SUMMARY AND CONCLUSIONS

In order to determine whether the transplantability, growth, and spread of the Brown-Pearce tumor might be influenced by factors reflected in the pretransplantation blood formulae, seventeen sets of experiments were carried out upon 195 young adult male rabbits, in apparent good health. Repeated determinations were made of the levels of the red blood cells, hemoglobin, platelets, total white blood cells, neutrophiles, basophiles, eosinophiles, and monocytes. Seventy-nine animals were discarded for no other reason than that one or more average blood factor levels were abnormal, and 8 animals were discarded for other reasons. The final analysis was therefore carried out on 108 animals. After the blood examinations had been concluded, the animals were inoculated intratesticularly with the Brown-Pearce tumor. Transplantation was unsuccessful, by the criteria set up, in 18 animals.

No relationship could be demonstrated between the success or failure of transplantation and the pretransplantation levels of the blood platelets, neutrophiles, basophiles, eosinophiles, monocytes, or lymphocytes. A statistically significant relationship was, however, demonstrated between the success and failure of transplantation and the pretransplantation levels of the hemoglobin, the red blood cells, and the total white blood cells. The hemoglobin and red cell levels were interrelated and the relationship of the red blood cell level to the success of transplantation seemed secondary to the hemoglobin level. No relationship of the red blood cell level to the success of transplantation could be demonstrated when the hemoglobin and total white blood cell levels were held constant.

The resistance of the rabbit to transplantation of the Brown-Pearce tumor was found to be associated with optimal or modal pretransplantation levels of the hemoglobin and the total white blood cells. When the average level of either the hemoglobin or the total white cells (independent variables) was not modal the animals were susceptible. Besides the hemoglobin and total white cell levels, the presence of a third unknown or unidentified factor (or factors) was postulated.

REFERENCES

1. Brown, W. H., and L. Pearce. Studies Based on a Malignant Tumor of the Rabbit. III. Intratesticular Transplantation and Clinical Course of the Disease. J. Exper. Med., **37**:799-810. 1923.
2. Casey, A. E. The Experimental Alteration of Malignancy With an Homologous Mammalian Tumor Material. I. Results With Intratesticular Inoculation. Am. J. Cancer, **21**:760-776. 1934.
3. Casey, A. E. The Reaction of Standard Breeds of Rabbits to the Brown-Pearce Tumor. Am. J. Cancer, **31**:446-456. 1937.

4. Casey, A. E., and L. Pearce. Studies on the Blood Cytology of the Rabbit. VIII. The Blood of Normal Rabbits as an Index of Their Resistance to a Transplantable Neoplasm. J. Exper. Med. **54**:475-492. 1931.
5. Casey, A. E., P. D. Rosahn, C. K. Hu, and L. Pearce. Hereditary Variations in the Blood Cytology of Normal Rabbits. Science, **79**:(N. S.) 189-190. 1934.
6. Casey, A. E., P. D. Rosahn, C. K. Hu, and L. Pearce. The Hemocytological Constitution of Adult Male Rabbits from Fifteen Standard Breeds. J. Exper. Med., **64**: 453-469. 1936.
7. DaFano, C. Zellulare Analyse der Geschwulstimmunitätsreaktionen. Ztschr. f. Immunitätsforsch., **5**:(Teil I, Orig.) 1-10. 1910.
8. Ehrlich, P. Experimentelle Carcinomstudien an Mäusen. Arb. Kgl. Inst. exper. Therap. Frankfort, **1**:77. 1906.
9. Fisher, R. A. On the Interpretation of χ^2 from Contingency Tables, and the Calculation of *P*. J. Roy. Statist. Soc., **85**:87-94. 1922.
10. Fisher, R. A. Statistical Methods for Research Workers. 4th ed., Oliver and Boyd, London, 1932.
11. Gaylord, H. R., G. H. A. Clowes, and F. W. Baeslack. An Anti-Carcinoma Serum. Med. News, **86**:91-92. 1905.
12. Hirschfeld, H. Discussion. Berl. klin. Wchnschr., **50**:80. 1913.
13. Hirschfeld, H. Ueber Heil- und Immunisierungsvorgänge an Tumortieren. Ztschr. f. Krebsforsch., **16**:93-95. 1919.
14. Koenigsfeld, H., and F. Kabierske. Ueber Blutveränderungen bei Tumormäusen. Med. Klin., **11**:646-648. 1915.
15. Murphy, J. B. The Lymphocyte in Resistance to Tissue Grafting, Malignant Disease and Tuberculous Infection: An Experimental Study. Monographs of the Rockefeller Institute for Medical Research, No. 21, New York, 1926.
16. Oba, K. On the Blood Changes of Mice by the Transplantation of Carcinoma. Sei-i-Kwai M. J., **43**:11-12. 1924.
17. Oba, K. On the Blood Features in Albino Rats Bearing Transplantable Carcinoma. Sei-i-Kwai M. J., **43**:13-14. 1924.
18. Oba, K. On the Changes of the Blood Picture in Sarcoma Bearing Rabbits. Sei-i-Kwai M. J., **46**:7-8. 1927.
19. Pappenheim, A. Blutbefunde bei malignen Tiertumoren. Folia haematol., **10**:II Teil, 393-394. 1910.
20. Pearce, L., and A. E. Casey. Studies in the Blood Cytology of the Rabbit. VII. Observations on Rabbits Inoculated with a Transplantable Malignant Neoplasm. J. Exper. Med., **53**:895-905. 1931.
21. Pearson, K. On the Criterion That a Given Series of Deviations From the Probable in the Case of a Correlated System of Variables Is Such That It Can Be Reasonably Supposed to Have Arisen From Random Sampling. Phil. Mag., Series V, **50**:157-175. 1900.
22. Price-Jones, C. Observations on the Blood and Bone Marrow of Healthy and of Carcinomatous Mice. Arch. Middlesex Hosp., London, **23**:56-72. 1911.
23. Price-Jones, C. The Cytology of the Blood in Sarcomatous Rats. Arch. Middlesex Hosp., London, **30**:162. 1913.
24. Ribbert, H. Das Karzinom des Menschen, sein Bau, sein Wachstum, seine Entstehung. F. Cohen. Bonn, 533 pp. 1911.
25. Rous, P. Metastasis and Tumor Immunity. Observations with a Transmissible Avian Neoplasm. J. A. M. A., **55**: 1805-1806. 1910.
26. Strong, L. C. Hemoglobin Levels in Various Degrees of Susceptibility to Spontaneous Tumors. Am. J. Cancer, **27**:500-509. 1936.
27. Strong, L. C., and L. D. Francis. The Blood of Female Mice (Breeders) of Cancer-Susceptible (A) and Cancer-Resistant (CBA) Strain. Arch. Path., **23**:202-206. 1937.
28. Woglom, W. H. Immunity to Transplantable Tumors. Critical Review. Cancer Rev., **4**:129-214. 1929.

Biocatalysts in Cancer Tissue

I. Cytochrome c*

K. P. DuBois, M.S., and V. R. Potter, Ph.D.

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison, Wis.)

(Received for publication January 12, 1942)

Since the time of Warburg's pioneer work on tumor metabolism considerable attention has been given to the oxidative and glycolytic quotients of various types of tumor tissue as contrasted with normal tissue. While there is fairly general agreement that tumor tissues exhibit high rates of anaerobic glycolysis, as well as aerobic glycolysis, Warburg's original idea of a low respiratory rate in tumors seems to have been modified, since many tumors show appreciable rates of oxygen uptake. Furthermore, the demonstration of high glycolytic rates in various nonmalignant tissues has introduced an element of uncertainty into the problem of classifying tissues as nonmalignant or malignant according to their metabolic quotients, and various investigators disagree as to the reliability of such classifications. Current opinion has been admirably discussed by Elliott (5) and by Burk (3).

It is now known, of course, that the metabolic quotients referred to above represent in fact the overall effect of several dozen competing and cooperating enzyme systems [1] and it must be apparent that any given metabolic quotient could be the result of a number of combinations of enzyme systems of widely varying activity. Thus a normal tissue and a tumor tissue might have the same rate of oxygen uptake which could result from a limited supply of substrates plus an excess of oxidative enzymes in the former and an excess of substrates and a limited supply of oxidative enzymes in the latter. Since, therefore, some of the similarities between tumor and normal tissue might be only apparent and might overlie more subtle changes in the individual biocatalysts which collectively produce a given metabolic quotient, we have initiated a program which has as its ultimate goal the definition of tumor tissue in terms of specific biocatalysts.

The first biocatalyst which we have studied in tumor tissues is cytochrome *c*. This compound appears to be one of the main connecting carriers in the transport of the hydrogen of the substrates to molecular oxygen, and a deficiency in this compound alone would be a sufficient explanation for the high aerobic glycolysis which occurs in most tumor tissue. That is, given a high rate of carbohydrate breakdown, a deficiency in cytochrome *c* would result in a high rate of lactic acid formation even in the presence of oxygen. Without attempting to answer the question of the specificity of aerobic glycolysis for tumor tissue we have attempted to get the simple facts regarding the concentration of cytochrome *c* in a variety of tumors. The types of tumors were selected with two points in mind; namely, diversity of etiology and possession of established metabolic characteristics.

Previous investigators have suggested the possibility of a deficiency of cytochrome *c* in some tumors. In none of the previous reports, however, was the study extended beyond one or two types of experimental tumors and then only a limited number of specimens were examined. Most of the reports have been of a qualitative nature. Holmes (7) reported that Jensen rat sarcoma and rat carcinoma No. 9 were deficient in cytochrome. Bierich, Rosenbohm, and Kalle (2) observed cytochrome bands in both normal and tumor tissues. von Euler (14) reported that Jensen rat sarcoma was deficient in cytochrome *c*. On the other hand Ball (1) stated that he observed an abundance of cytochrome *c* in Jensen rat sarcoma and Stern was recently quoted (13) to the effect that cytochrome *c* can be readily observed in Yale mouse tumor No. 1. The above reports were all based on qualitative spectroscopic studies which give only comparative indications of the concentration, and cannot be used with a high degree of reliability to establish quantitative differences.

In 1939, Junowicz-Kocholaty and Hogness (8) developed a quantitative procedure and reported the content of cytochrome *c* in a human liver carcinoma as only 3 μgm. per gm. of fresh tissue. They found no cytochrome in a specimen of human fibromyoma. In the same year Stotz (13) devised a new method for the determination and reported that 3 specimens of rat tumor R-256 contained an average of 3 μgm. of cytochrome *c* per gm., while 3 specimens of R-39 and one specimen of spontaneous tumor contained 5 and 2 μgm., respectively.

From the information reported up to the present time it is impossible to arrive at any definite conclusion regarding the concentration of cytochrome *c* in cancer tissue although the latter two reports certainly indicate that a deficiency of cytochrome *c* might be a general finding in tumor tissue. We felt that this conclusion would be of such fundamental importance

* This investigation was aided by a grant from The Jonathan Bowman Fund for Cancer Research.

[1] Some of these reactions have been illustrated diagrammatically by one of the authors (10).

that it would be desirable to attempt to make a generalization.[2]

MATERIALS AND METHODS

The cytochrome *c* content of the tissues was determined by the method of Potter and DuBois (12). The method employs a specific enzymatic reduction of cytochrome *c*, which is then measured quantitatively by means of a photoelectric spectrophotometer. The method was tested for accuracy by recovery experiments in which pure cytochrome *c* was added to tumor tissue and recovered to the extent of 93 per cent or better. From 5 to 10 gm. of tumor tissue were used for each determination, which was carried out in duplicate using pooled samples of tumor tissue. Each analysis thus generally represents the average of from 3 to 10 rats or mice. Special care was taken to avoid necrotic tissue as well as the normal tissue surrounding the tumor tissue. Histological examinations were made from time to time to assure the use of good tumor tissue for the analyses. Analyses of a sufficient number of specimens of each type of tumor were made so that a reliable average value for each type of tumor could be obtained. All tumors studied were of known etiology since values obtained with tumors of unknown origin are of little value in an experimental study of various constituents owing to lack of reproducibility. Experimental tumors of widely different etiology were studied so that any differences in tumors induced by different means might be noted.

RESULTS

The results of the analyses of tumors from several hundred animals, including rats, mice, and chickens are shown in Table I. Values obtained with a number of normal tissues are given in Table II for purposes of comparison. Nine types of tumor are represented, many of which have been studied extensively from the metabolic standpoint. The etiological factors include ultraviolet light, estrogenic substances, virus, subcutaneously injected 2,2′-azonaphthalene, and orally ingested dimethylaminoazobenzene (butter yellow). The average value in every case has been remarkably consistent within the range of from 10 to 20 μgm. of cytochrome *c* per gm. of fresh tissue. In fact the highest individual value in the entire series was only 26 μgm. per gm. No values have been discarded. Of some individual interest are the cases of Yale mouse tumor No. 1 and Jensen rat sarcoma, both of which had been stated to contain appreciable amounts of cytochrome. These statements are undoubtedly correct in the sense that the presence of cytochrome can be demonstrated in these tumors. However, the quantitative data make it possible to compare the tumors with normal tissues on an absolute basis. In the case of the tumors induced by butter yellow, there were a number of cases in which the tumors were too small and too scattered to be dissected out. In these cases the entire lobe was analyzed and reported as tumorous liver. The results fell between the values for liver tumors and normal livers, as might be expected.

[2] A preliminary report has appeared (11).

TABLE I: THE CYTOCHROME *c* CONTENT OF EXPERIMENTAL TUMORS

Tumor *	Etiology	Number of analyses	Cytochrome *c* in γ/gm.† Minimum	Maximum	Average
Flexner-Jobling rat carcinoma	Spontaneous	14	8	15	12
Walker 256 rat carcinoma	Spontaneous	11	5	11	9
Jensen rat sarcoma	Spontaneous	7	9	19	12
Yale No. 1 mouse tumor	Estrin	12	10	20	16
Ultraviolet ear tumor, mouse	Ultraviolet irradiation	8	5	20	11
Rous chicken sarcoma	Virus	12	7	14	12
Mouse tumor	2,2′-Azonaphthalene	7	12	19	15
Mouse mammary tumors	Spontaneous	6	11	16	14
Rat liver tumor	Butter yellow	11	13	26	20
Tumorous rat liver	Butter yellow	8	44	75	61

* The first seven types of tumors were obtained by transplanting existing strains. Several generations of tumors were analyzed in each case.
† Based on fresh tissue. Assumes molecular weight of 16,500 for cytochrome *c*. See (12).

TABLE II: CYTOCHROME *c* CONTENT OF NORMAL RAT TISSUES *

Tissue	Cytochrome *c*, γ/gm.	Tissue	Cytochrome *c*, γ/gm.
Heart	371	Brain	50
Kidney, whole	247	Spleen	43
Skeletal muscle	97	Lung	21
Liver	90		

* Data from Potter and DuBois (12). Each figure is the average from 10 rats.

In the case of the butter yellow tumors, the marked difference between the cytochrome content of the tumors as compared with normal liver led us to attempt a study of the cytochrome content of precancerous livers. Since the production of liver tumors required the feeding of the dye for about 6 months it seemed that any cytochrome changes in the precancerous stage might be observed by following the liver cytochrome from the beginning of the experiment to the time of the appearance of tumors. We believed that if the decrease in cytochrome were a progressive change which preceded cancer formation it could be observed

by this type of experiment. For this study, 40 rats were placed on a ration containing 0.06 per cent butter yellow.[3] At various intervals throughout the experiment rats were killed and the cytochrome content of their livers was determined. The rats killed were those which had gained the most together with an equal number of those which had gained the least in body weight. This was done as an arbitrary correction for the variation in reaction to the dye. The data showed little if any significant lowering of the cytochrome *c* in the livers prior to the time that tumors were grossly apparent. After the appearance of tumors the tumorous livers definitely contained less cytochrome than normal livers, in agreement with our previous experience. Difficulties are encountered in an experiment of this type in that the time of appearance of tumors differs widely among individual animals on the same ration. The experiment was thus weakened by the fact that we were unable positively to affirm that the livers examined were precancerous. However the fact that no significant lowering of the amount of cytochrome was detected in any livers except those which contained obvious tumors makes it appear likely that the cytochrome concentration is not decreased in the precancerous phase.

DISCUSSION

The results suggest that a deficiency of cytochrome *c* may be a characteristic of tumors in general. The apparent contradictions in the literature regarding the concentration in various tumors are probably due to the fact that many of the measurements were not quantitative. The reports of quantitative determinations are in harmony with our own except that they report appreciably lower values for tumor tissues than we have found.

It may be asked whether the levels of cytochrome *c* which we have observed in tumor tissue constitute a deficiency, since a normal tissue such as lung contains very little more than the tumors. However the rate of carbohydrate breakdown must be taken into consideration, since it is the energy requirement of the tissue which sets the requirement for aerobic enzymes. Lung tissue has a low rate of carbohydrate breakdown and thus has no great need for cytochrome. When compared with active tissues the cytochrome content of tumors appears to be definitely low, and it seems reasonable to consider tumors as active tissues. Probably the best comparison can be made between liver and liver tumor, and in this case the latter contains only about one-fifth as much cytochrome as normal liver. If the higher cytochrome level in normal liver be regarded as an excess for a margin of safety one must ask why the tumor is not similarly equipped.

The apparent deficiency in cytochrome *c* may be taken as further support for Warburg's original contention (15) that in tumors there exists a type of metabolism (glycolytic) which is better able to withstand anaerobic conditions than are the adjacent normal tissues. By an extension of this concept any factor which decreases the effectiveness of the aerobic mechanism should damage the potential cancer cell relatively less than the adjacent normal cells and thus facilitate carcinogenesis. More specifically, if the energy requirements in the case of cancer tissue are less dependent upon cytochrome *c* than in the case of normal tissue, then factors which adversely affect the functioning of cytochrome *c*, either directly or indirectly (by acting on systems which oxidize or reduce cytochrome *c*), should damage normal cells more than potential cancer cells.

There are at present indications that succinic dehydrogenase, which is another unit in the aerobic mechanism, is also deficient in certain types of tumor tissue. By contrast, it has been reported that at least one enzyme[4] necessary for *glycolysis* is no lower in liver tumor than in normal liver (6). Confirmation and extension of these two observations would lend further support to the concept outlined above. It might also be anticipated that various carcinogenic chemicals might be found to be more toxic to the aerobic enzymes than to the glycolytic mechanism.

SUMMARY

1. The cytochrome *c* content of nine kinds of experimental tumors has been determined.

2. The tumors contained from 10 to 20 μgm. of cytochrome *c* per gm. of fresh tissue, regardless of etiology.

3. In the case of liver tumors produced by feeding butter yellow, the tumors contained about one-fifth as much cytochrome as normal liver.

4. The results seem to lend further support for Warburg's idea that tumors possess a type of metabolism which is better able to withstand anaerobic conditions than is normal tissue.

The authors are indebted to Drs. K. G. Stern and P. P. Cohen for the Yale mouse tumor No. 1, to Drs. Carl Voegtlin and J. B. Thompson for the Jensen rat sarcoma, to Dr. W. L. Wasley for the 2,2′-azonaphthalene-induced mouse sarcoma, to Drs. P. F. Clark and A. F. Rasmussen for the Rous chicken sarcoma, and to Dr. H. P. Rusch for kind advice and material help.

[3] The diet consisted of casein 10, cerelose 76, salts 4, Primex 5, cod liver oil 1, and yeast 4. Improved diets for this type of work have since been developed in this laboratory (9).

[4] The enzyme was stated to be amylase but it appears likely that it was in reality phosphorylase (4) which is an essential enzyme in glycolysis. The difference between the two enzymes is that the former splits glycogen by hydrolysis while the latter splits glycogen by phosphorolysis.

REFERENCES

1. Ball, E. G. Discussion in Cold Spring Harbor Symposia on Quantitative Biology, **7**:119. 1939.
2. Bierich, R., A. Rosenbohm, and K. Kalle. Untersuchungen über das Zustandekommen der bösartigen Geschwülste. 4. Über den Gehalt von normalen und Krebsgeweben an Milch säure, Cytochrom und Glutathion. Ztschr. f. physiol. Chem., **164**:207-217. 1927.
3. Burk, D. Discussion on Tumor Respiration. To be published in A Symposium on Respiratory Enzymes, University of Wisconsin Press, Madison, Wis.
4. Cori, G. T., and C. F. Cori. Enzymatic Breakdown of Glycogen in Liver Extracts. Proc. Soc. Exper. Biol. & Med., **39**:337-338. 1938.
5. Elliott, K. A. C. Discussion on Tumor Respiration. To be published in A Symposium on Respiratory Enzymes, University of Wisconsin Press, Madison, Wis.
6. Greenstein, J. P., W. F. Jenrette, and J. White. The Relative Activity of Xanthine Dehydrogenase, Catalase, and Amylase in Normal and Cancerous Hepatic Tissues of the Rat. J. Nat. Cancer Inst., **2**:17-23. 1941.
7. Holmes, B. E. Oxidative Mechanisms in Tumor Tissue. I. The Anaerobic Habit of Tumor Tissue. Biochem. J., **20**:812-816. 1926.
8. Junowicz-Kocholaty, R., and T. R. Hogness. The Spectroscopic Determination of Cytochrome C and its Distribution in some Mammalian Tissues. J. Biol. Chem., **129**:569-575. 1939.
9. Miller, J. A., D. L. Miner, H. P. Rusch, and C. A. Baumann. Diet and Hepatic Tumor Formation. Cancer Research, **1**:699-709. 1941.
10. Potter, V. R. The Mechanism of Hydrogen Transport in Animal Tissues. Medicine, **19**:441-475. 1940.
11. Potter, V. R., and K. P. DuBois. The Determination of Cytochrome *c* in Tumor Tissue. Proc. Am. Soc. Biol. Chem., J. Biol. Chem., **140**:cii-ciii. 1941.
12. Potter, V. R., and K. P. DuBois. The Determination of Cytochrome *c*. J. Biol. Chem., **142**:417-427. 1942.
13. Stotz, E. The Estimation and Distribution of Cytochrome Oxidase and Cytochrome C in Rat Tissues. J. Biol. Chem., **131**:555-566. 1939.
14. von Euler, H. Biochemische Krebsprobleme. Deutsche med. Wchnschr., **64**:1712-1717. 1938.
15. Warburg, O., K. Posener, and E. Negelein. Über den Stoffwechsel der Carcinomzelle. Biochem. Ztschr., **152**: 309-345. 1924.

A Tumor of the Adrenal Medulla in a Castrated Male Rat

W. C. Hueper, M.D., and Gustav J. Martin, Sc.D.

(From the Warner Institute for Therapeutic Research, New York, N. Y.)

(Received for publication January 7, 1942)

Adrenal tumors in man are of interest not only because of their relative rarity, but also because of their relation to hypertension and thus to arteriosclerosis and because of their influence upon the secretion of the sex hormones resulting in precocious "puberty" and masculinization.

The occurrence of spontaneous neoplasms of these glands in animals is apparently highly exceptional. Peyron (3) observed adrenal paragangliomas in 3 cows, 1 sheep, and 5 horses. Löwenthal (2) recorded the presence of several cortical adenomas and of a medullary pheochromocytoma in mice. Three morphologically similar tumors in the medulla of the adrenals of mice, diagnosed as mesotheliomas, as well as one cortical adenoma in these animals, were reported by Slye, Holmes, and Wells (4).

The experimental production of blastomatoid and blastomatous conditions in these glands was accomplished in recent years by various methods. Staemmler (6) noted the occurrence of adenomatoid proliferations in the adrenal medulla of rats subjected over prolonged periods to subcutaneous injections of nicotine, a substance which activates the release of adrenalin. Spiegel (5) observed the appearance of cortical tumors in old male guinea pigs which had been castrated at an early age. Similar neoplastic reactions were found by Woolley, Fekete, and Little (7, 8) in female mice ovariectomized immediately after birth. Gardner (1) observed the occurrence of tumorous conditions in ovariectomized mice receiving intravaginal instillation of benzpyrene dissolved in oil, in untreated ovariectomized mice, or in mice receiving estrogens for prolonged periods. The untreated ovariectomized mice were castrated from 5 to 9 weeks after birth.

The following communication deals with an adrenal tumor found at autopsy in a male rat castrated at the age of 1 month and maintained on a vitamin E-deficient diet for a subsequent period of 12 months. This animal was one of a series of 30 similar rats which were killed when 13 months old. While the adrenals of these rats were in general grossly and microscopically normal, the left adrenal gland of this particular animal was found at autopsy to be a round, white-yellow node surrounded by a smooth capsule and measuring 1 cm. in diameter. The cut surface was yellowish-white and homogeneous. The other internal organs were normal.

The histological examination of the tumor showed that it consisted mainly of densely packed, ill-defined, round or irregularly shaped cells having a round, moderately chromatic nucleus. Mitotic figures were rare. In some portions there were areas of larger, polygonal cells with a loose, pink-stained cytoplasm. Interspersed in this cellular matrix were strands of elongated, large cells arranged in loose bundles. The oval-shaped and irregular-sized nuclei were often hyperchromatic, while the cytoplasm was relatively abundant and deeply pink-stained (Fig. 1). The stroma was scanty and consisted mainly of thin-walled capillaries. The neoplasm grew expansively compressing the atrophic and vacuolated cellular cortical tissue, which covered the tumor like a cap. The demarcation between the two tissues was indistinct. There was no

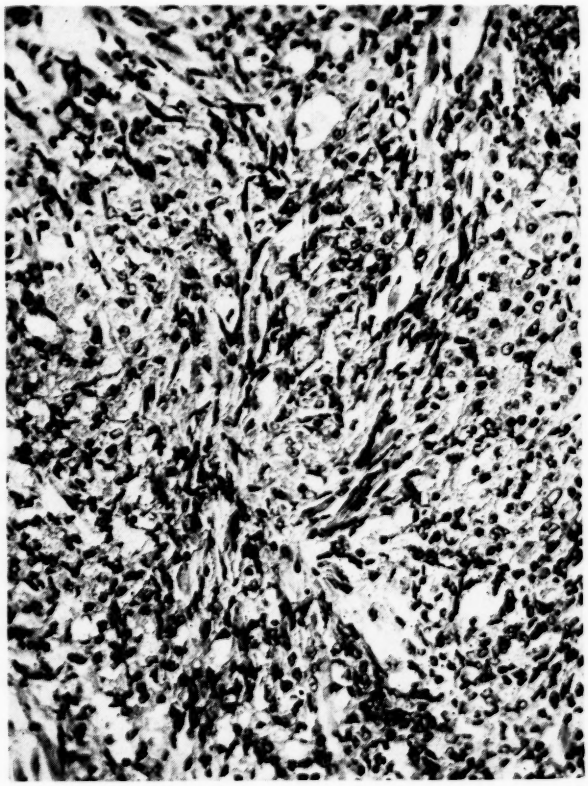


FIG. 1.—Photomicrograph of section of tumor of adrenal medulla showing ill-defined and irregular groups of larger lightly stained cells and of smaller more intensely stained cells of indistinct outlines and oval or round shape with transitions into elongated spindle-shaped cells, which are often arranged in bundles. Hematoxylin and eosin stain. Mag. × 200 (approx.).

invasion of the surrounding tissue. Sections stained for chromaffinic material by the method of Schmorl (Giemsa staining) were free from any green-colored, chromaffinic cellular granulation.

The examination of the other internal organs revealed moderate arteriolosclerotic lesions in the myocardial, pulmonary, and renal tissues. The walls of the cerebral vessels were often thickened and hyaline, and occasionally surrounded by small hemorrhages. Small degenerative and fibroblastic foci were present in the myocardium.

SUMMARY AND CONCLUSIONS

The adrenal tumor described was found in one of 30 castrated male rats kept on a vitamin E-deficient diet and was apparently of medullary origin. The location of the tumor in the gland, the presence of nervous tissue elements in the neoplastic parenchyma, and also, to a certain extent, the occurrence of arteriolosclerotic lesions in various internal organs support this diagnosis. In view of the absence of chromaffinic matter in the cells of the neoplasm, the diagnosis of ganglioneuroma of the adrenal seems to be justified.

It is uncertain whether or not the development of this blastoma is causally related to the endocrine and vitamin disturbances experimentally produced in this rat. Investigations now under way may clarify this question, which is important, as Gardner concluded that the adrenal tumors observed in ovariectomized mice are of cortical derivation and originate from the zona glomerulosa; *i.e.,* remote from the androgenic zone.

REFERENCES

1. Gardner, W. U. Estrogenic Effects of Adrenal Tumors of Ovariectomized Mice. Cancer Research, **1**:632-637. 1941.
2. Löwenthal, K. Nebennieren. Published in Jaffé, R. Anatomie und Pathologie der Spontanerkrankungen der Kleinen Laboratoriumstiere. J. Springer, Berlin, p. 363. 1931.
3. Peyron, A. Le paragangliome surrénal et son syndrome cardiovasculaire. Bull. Assoc. franç. p. l'étude du cancer, **19**:618-650. 1930.
4. Slye, M., H. F. Holmes, and H. G. Wells. Studies on the Incidence and Inheritability of Spontaneous Tumors in Mice. XVII. Primary Spontaneous Tumors in the Kidney and Adrenal in Mice. J. Cancer Research, **6**:305-336. 1921.
5. Spiegel, A. Über das Auftreten von Geschwülsten der Nebennierenrinde mit Vermännlichender Wirkung bei Frühkastrierten Meerschweinchenmännchen. Virchows Arch. f. path. Anat., **303**:367-393. 1939.
6. Staemmler, M. Die chronische Vergiftung mit Nicotin. Ergebnisse experimenteller Untersuchungen an Ratten. Virchows Arch. f. path. Anat., **295**:366-393. 1935.
7. Woolley, G., E. Fekete, and C. C. Little. Differences Between High and Low Breast Tumor Strains of Mice When Ovariectomized at Birth. Proc. Soc. Exper. Biol. & Med., **45**:796-790. 1940.
8. Woolley, G., E. Fekete, and C. C. Little. Effects of Castration in the Dilute Brown Strain of Mice. Endocrinology, **28**:341-343. 1941.

Observations on the Colloidal Vanadate Reaction (Bendien Reaction) in a Series of Cases of Carcinoma of the Cervix*

Arthur A. Gemmell, M.C., T.D., M.D. (Cantab.) F.R.C.S.E., F.R.C.O.G.

(From the Department of Obstetrics and Gynaecology, the University of Liverpool, Liverpool, England)

(Received for publication November 3, 1941)

The colloidal vanadate reaction, or Bendien reaction (1), has been the subject of much controversy and unfavorable criticism.

During the years 1929-37, 151 cases of carcinoma of the cervix have been treated and followed up. The full results of this follow-up are to be published in a later paper. While this has been in progress observations on the colloidal vanadate reaction (C.V.R.) have been made on some of the cases and the purpose of this paper is to record these in detail. The observations appear to furnish interesting information and it is hoped that their publication will lead to a similar investigation in groups of other cases such as carcinoma of the stomach or of the breast.

It is recognized that this test is not diagnostically specific for cancer. In the present series, however, this fact is not of importance, for we are dealing with a group of patients all suffering from proved malignant disease. Histological confirmation is available in every case except one. The tests were carried out by Cronin Lowe who had no knowledge of the clinical condition of the patient at the time the blood was sent to him. This information was communicated to him only after receipt of his report.

THE TEST

Cronin Lowe (2) has described fully the "three-phase" modification of the colloidal vanadate reaction. The following brief summary explains the essential points, but anyone who desires to repeat the tests is advised to consult the original article.

Five to 10 cc. of blood are collected aseptically and with precautions against hemolysis into a dry sterile test tube and allowed to clot. The serum is divided into 3 portions:

The first portion is untreated and designated A.
The second portion is heated to 56° C. for 30 minutes and designated B.
The third portion is extracted with ether and designated C.

A series of solutions of colloidal vanadate is prepared in the following manner: 20 cc., 21 cc., 22 cc., and so on up to 30 cc. of decinormal sodium orthovanadate are each made up to a volume of 200 cc. by adding decinormal acetic acid.

The untreated portion, A, of the serum is first tested against this series and the weakest dilution of vanadate which gives a precipitate is noted. This is recorded as 20, 21, and so on, according to the dilution in the tube. Throughout this paper this result with the A portion of the serum alone is referred to as "initial flocculation" or I.F.

The second and third portions of the serum are then subjected to precipitation by this dilution of orthovanadate solution, and the amount of precipitate produced in each of the three portions, A, B, and C, is measured in interferometer units. Using the letters, A, B, and C now for the number of interferometer units of precipitate, Cronin Lowe works out a fraction $\frac{C-A}{A-B}$. He maintained originally that when this fraction was greater than unity the result should be classified as "malignant," when less than unity "innocent," and when the quotient obtained by dividing the numerator by the denominator is between 0.8 and 1.2 "indifferent." Throughout this paper this result is referred to as "the fraction."

Cronin Lowe's article states that the blood should be taken from the patient in a fasting condition. Oliver (3) points out the difficulty of obtaining fasting blood from patients coming up for a follow-up visit, and in this series it has been found impracticable to impose the fasting condition on patients. This omission must, to some extent, vitiate the later conclusions on the value of the fraction but it should be noted that the fasting condition chiefly influences Cronin Lowe's B and C portions and does not influence the initial flocculation of the A portion.

Price (4) has produced evidence to show that the point of initial flocculation is determined by a balance between (a) the total protein concentration, (b) the albumin/globulin ratio, and (c) the amount of lipoid present in the serum. He found that an increase in

* The manuscript of this paper was accompanied by 44 graphs showing reactions in individual cases. It is regretted that space was not available for the reproduction of all of these figures.—EDITOR.

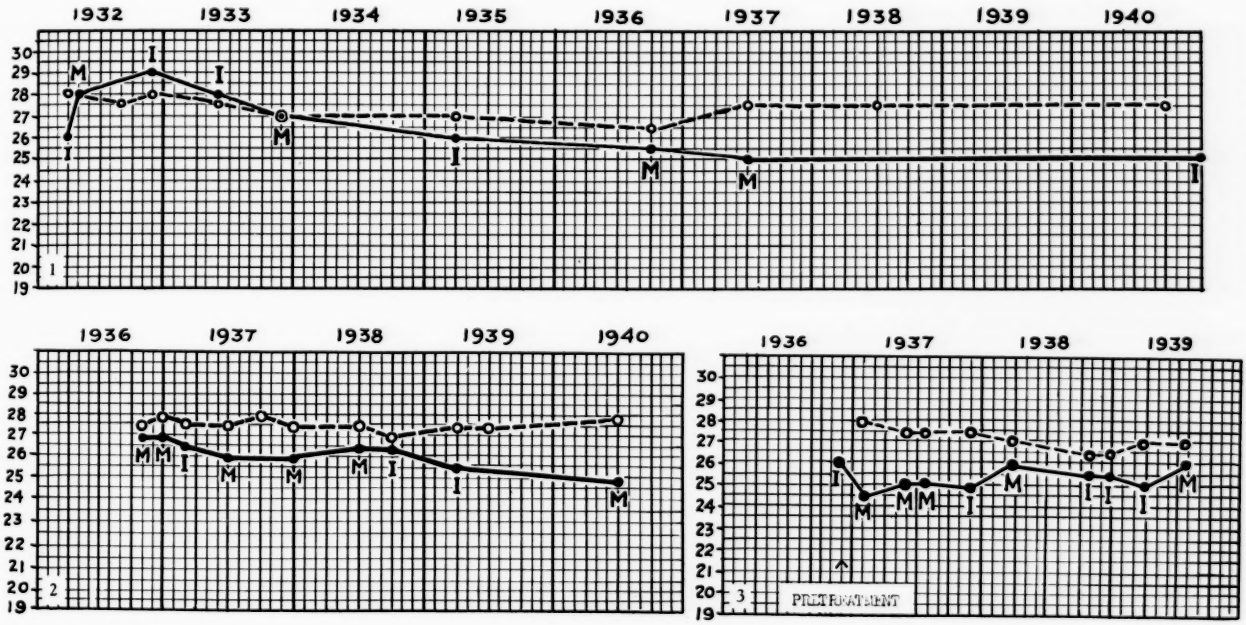
the serum globulin, with consequent alteration in the albumin/globulin ratio, was usually associated with an increase in the amount of lipoid present and when these two factors occurred together the initial flocculation was obtained with the lower dilutions.

METHOD OF ASSESSING RESULTS

The results of the tests are assessed in two groups: 1. repeated observations during the follow-up period and 2. a single observation before treatment commenced. The former should be assessed in terms of their value in prognosis; the latter in prognosis and diagnosis. In each group there are two parts of the test to evaluate (a) the initial flocculation in the untreated serum and (b) the fraction. It may be of interest to the reader to know that the author had made no attempt to assess the significance of the tests recorded until the preparation of this paper was undertaken.

Repeated observations during the follow-up period.—There are available 44 records of patients who have had four or more tests during periods ranging from just over 1 year up to 8 years. Forty-three of these patients were treated with radium and one by Wertheim's operation. In Figs. 1, 2, 3, and 6 the results of the C.V.R. tests are represented graphically for 4 of these patients, each graph being accompanied by an estimate of the clinical condition.

In these figures the initial flocculation is shown by the continuous line graph. The letter I beside a point denotes an innocent or indifferent fraction, the letter M a malignant one. These letters denote the character of the fraction only and play no part in determining the position of the point on the graph. The clinical condition is depicted by the dotted line graph in which each sympton or sign of possible recurrence is shown by recording one square below, or, if very marked, two squares below an arbitrary base line of health shown in all the graphs at the abscissa level of 28. The symptoms and signs and the number of squares allocated to them are listed in a footnote.[1]



Graphs of C.R.V. reactions, solid line; estimate of clinical condition,[1] broken line. I = innocent reaction. M = malignant reaction. Values of initial flocculation are shown on the ordinate.

FIG. 1.—Patient in Stage II; treated July, 1930; alive and well. (Author's Graph 1, No. 14.)
FIG. 2.—Patient in Stage I; treated May, 1936; alive and well. (Author's Graph 15, No. 102.)
FIG. 3.—Patient in Stage I; treated October, 1936; alive and well. (Author's Graph 17, No. 114.)

[1] METHOD OF PLOTTING CLINICAL FINDINGS ON PROGRESS GRAPHS

The horizontal line at abscissa level 28 is used as a base line representing absence of symptoms and signs.

When clinical signs appear they are plotted *below* this base line.

1 Square, symptoms: severe constipation; frequency of micturition; dysuria; slight vaginal discharge; hematuria without discovered cause; occasional rectal hemorrhage; slight bleeding on coitus; pain in lumbar region; pain over the kidney; pain in the back.

1 Square, signs: cervix a little nodular; slight thickening in region of cervix; slight thickening in one broad ligament; slight thickening in parametrium posteriorly; slight thickening of posterior vaginal wall; small nodule in posterior fornix.

2 Squares, symptoms: frequency of micturition and constipation; flatulence and edema of ankles; pain in the buttock and dyspepsia; abdominal pain and some diarrhea; pain in the back

It is frankly recognized that it is really impossible to represent clinical observations mathematically, but the defense of the method adopted is that no other way could be devised to demonstrate the clinical picture visually. The letter R (Fig. 6) denotes that there was definite evidence of clinical recurrence. Death is shown by an arrow and the word "Died."

In studying the graphic representations of the clinical progress of these 44 cases, on the one hand, and the results of the colloidal vanadate reaction on the other, it seemed that the value of this test could best be assessed by:—

1. Noting whether there was any sharp and significant difference between the C.V.R. graphs of those patients who are alive at the time of reporting and the graphs of those who have died while under observation.

2. Noting the degree of correspondence between the actual C.V.R. graph record and the observed clinical progress of each individual case.

A. Comparison of Graphs of Surviving and Fatal Cases

In order to obtain a clear picture under this heading a composite graph has been constructed for the 23 living cases and another for the 20 who have died (Figs. 4 and 5).

Patients alive.—Fig. 4 comprises 164 observations on 23 patients (including one patient who died of intercurrent disease, although apparently doing well as far as the carcinoma of the cervix was concerned). The initial flocculation figure is plotted vertically. The left end of the graph is the date of the commencement of treatment and each square horizontally represents one month. The vertical lines divide the time into periods of 3 months for convenience. The mean is an almost straight line falling very slowly towards its right. It will be noted that there are only 5 readings above 28, all of which are in the first 3 years after treatment; there are only 6 below 25 and of these 5 appear after the 3rd year from the time of treatment.

These readings and the general shape of the graph agree with the known clinical facts that of the patients who survive for 3 years after treatment a proportion die before the end of the 5th year; and of those who survive 5 years a smaller proportion die before the 10th year.

It is of interest to record that the average reading of all these cases during the first 3 years after treatment is 26.3. Bendien's original Tube 6, which he accepted as the healthy reading, would be represented in the present method of recording as 27.

From these observations it is concluded that: 1. For a patient who is remaining well there appears to be a zone of initial flocculation between 25 and 28 which may be regarded as satisfactory. 2. For a patient who is remaining well a graphic record of repeated initial flocculation estimations may be expected to remain practically horizontal.

Patients dead.—There are 20 records comprising 105 observations. Fig. 5 is a composite graph for this group. The right end of the graph is the date of death and the other factors are recorded as in Fig. 4. It will be seen that during the last year of life there are 17 readings of 25 or over, while there are 29 readings below 25 and of these low readings 24 are at or under 23.

The curve begins to fall about 2 years before death and falls quite steeply in the last year of life. This fall would be even steeper were it not for the three very high readings in the last 18 months of life, all of which form the record of one patient (Fig. 6).

The conclusions drawn from these observations are: 1. Patients who are doing badly may be expected to have initial flocculation values below 25 and many of them will be in the region of 23 or below. 2. The graph for patients who are doing badly is likely to be a falling curve.

The striking difference between these two composite graphs (Figs. 4 and 5) establishes without any doubt that serial estimations of the initial flocculation of the C.V.R. have definite prognostic value in a large proportion of treated cases of carcinoma of the cervix. This conclusion gains confirmation from the examination of the records according to the second method suggested above.

---

and melena; rectal pain and hemorrhage; several rectal bleedings; several vaginal bleedings.

2 Squares, signs: definite nodular swelling in one broad ligament; parametrial thickening in two areas; some thickening in vaginal wall and one area of parametrium.

2 Squares, symptoms and signs: some bleeding and slight unilateral thickening; pain in the back and thickening in the uterosacral ligament; pain in the leg and some unilateral thickening; slight bleeding on examination and some roughening of the cervix; mild digestive symptoms and a nodule in the posterior vaginal wall; escape of blood and pus on examination and slight thickening in one broad ligament.

3 Squares, signs: considerable thickening on lateral pelvic wall; considerable thickening in posterior area of parametrium.

3 Squares, symptoms and signs: pain in the back, rectal hemorrhage, thickening in one broad ligament; frequency of micturition, constipation, and edema of the legs; discharge and thickening in both broad ligaments; abdominal pain; considerable thickening in one broad ligament.

4 Squares: phlebitis, abdominal pain, mass of glands in kidney region.

Increases in signs and/or symptoms from time of previous examination are shown thus: slight increase, 1 square; moderate increase, 2 squares; marked increase, 3 squares.

Correction

See: Gemmell, A. A. Observations on the Colloidal Vanadate Reaction (Bendien Reaction) in a Series of Cases of Carcinoma of the Cervix. Cancer Research, **2**:296-302. 1942.

The following graph and legend should replace those published on page 299:

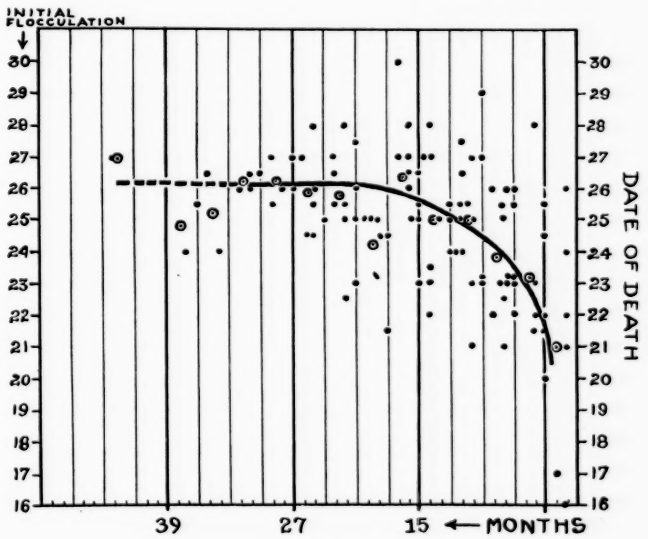


FIG. 5.—Composite graph of 105 observations of C.R.V. reactions in 20 patients who have died.

In the legend for Fig. 4 on page 299, the following words should be deleted: "and estimates of clinical condition[1]."

B. Comparison of Clinical Progress with C.V.R. Results

In this investigation the 44 cases are divided into two groups according to graphic records of the assessment of their clinical progress based on careful, though arbitrary, values assigned to a large series of clinical observations. These observations and the mathematical readings and in general it will be observed that the two graphs correspond in form and trend. All show a more or less horizontal initial flocculation graph, the majority having values between 25 and 28.

The undermentioned graphs deserve special comment.

Graphs 15 and 17 (Figs. 2 and 3) are of particular

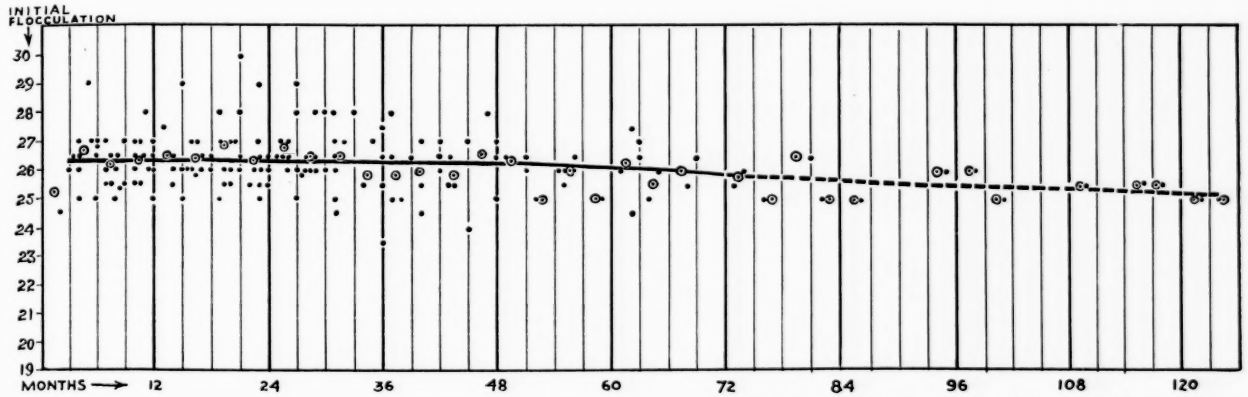


Fig. 4.—Composite graph of 164 observations of C.R.V. reactions and estimates of clinical condition [1] in 23 living patients.

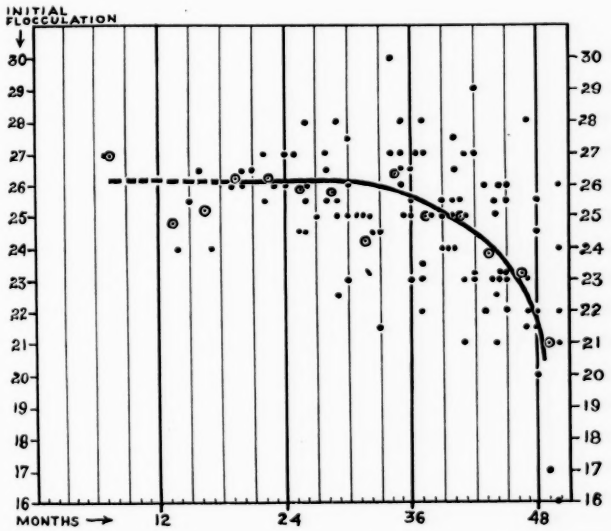


Fig. 5.—Composite graph of 105 observations of C.R.V. reactions and estimate of clinical condition [1] in 20 patients who have died.

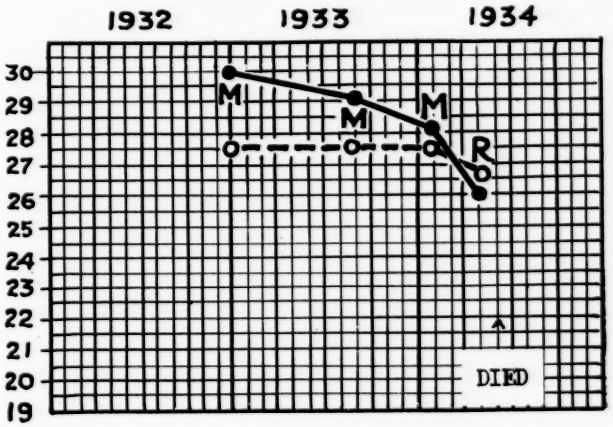


Fig. 6.—Graph of C.R.V. reaction, solid line; estimate of clinical condition,[1] broken line. M = malignant reaction. R = recurrence. Values of initial flocculation are shown on the ordinate. Patient in Stage II; treated July, 1932; died. (Author's Graph 40, No. 40.)

significance attached to each are set out in the footnote.[1] The two groups comprise (a) 23 cases who are doing well and (b) 21 whose clinical progress was bad. The clinical condition is assessed as "bad" either when there is obvious evidence of recurrence or metastasis, or when the graph shows a persistent fall of 3 or more squares below the arbitrary "healthy" level.

(a) Three clinical-progress-graphs representative of the 23 cases which are regarded as satisfactory or good are shown in Figs. 1-3 (dotted lines). Each of these graphs is accompanied by the graphic record of C.V.R. interest. Each of these patients at one time (Graph 15, Fig. 2, in October, 1938 and Graph 17, Fig. 3, in August, 1937) presented signs and symptoms which might have been interpreted as a recurrence or as a delayed rectal reaction according to Todd (5). Both were treated for the latter condition and the subsequent clinical course showed this view to be correct. It will be seen that in both cases the initial flocculation value remained within the normal zone.

It should be noted that all the high readings (*i.e.*, above 28) (Fig. 4) occur roughly in the first 2 years

following treatment. There is no explanation offered of the significance of this.

(b) Fig. 6 is representative of the graphs of 20 patients who are doing badly clinically. All the graphs of these cases show a downward trend in the curve and nearly all fall below the 25 to 28 level of initial flocculation.

Graph 40 (Fig. 6) shows a typical falling curve but the values are all high and stand out in the composite graph, Fig. 5. Reference to the section on the pretreatment readings, which follows later in the paper, shows that some patients have a high pretreatment reading and it may be that there are some individuals whose normal level is high. Bendien drew attention to these high readings and suggested that they might be related to a "predisposition to cancer." In this case the tendency of the graph would be of more importance than the actual value of the readings. Again, reference to Fig. 4 shows that high values are seen in the first 2 years following treatment and this patient (Fig. 6) died 16 months from the commencement of treatment.

These observations may be summarized as follows:—

For the 23 patients who appear to be doing well clinically, 21 (91.3 per cent) of the initial flocculation graphs are approximately horizontal and remain within the zone 25 to 28. Two of the initial flocculation graphs have fallen below the normal zone and more time is necessary to assess the significance of this.

For the 21 patients who are doing badly clinically, 14 (70 per cent) show a falling initial flocculation graph going below the level of 25; 16 (80 per cent) show a falling type of initial flocculation graph but 2 of these do not go below the 25 level. Seventeen (85 per cent) show a falling type of initial flocculation graph but 3 of them do not fall below the 25 level; and of these 3 one has an unexplained terminal rise.

An analysis of the 14 cases which are typical of this group whose progress is unsatisfactory shows that the initial flocculation value fell below 25 before a recurrence was diagnosed in 6 instances (43 per cent), with the recurrence in 1 instance (7 per cent), and after the recurrence was diagnosed in 7 cases (50 per cent). It is only fair to add, however, that in 2 of the last 7 cases no blood was taken for examination at the visit at which recurrence was diagnosed.

No facts can be obtained from a careful scrutiny of the follow-up records and death certificates of the 20 fatal cases which might offer an explanation of why in some cases the initial flocculation falls before recurrence can be diagnosed, in others at the time of recurrence, and in still others after this diagnosis has been made.

EVALUATION OF THE FRACTION

If the graphs are examined it will be noted that there is very considerable variation in the fraction according as it is classified I or M. This variation is seen in practically every record and there is no relationship between the numerical value given to the initial flocculation result and the pathological value of the fraction. With high initial flocculation values we find sometimes an I fraction and sometimes an M. The same is true of low values. Again, a patient may have had a series of M fractions and then shortly before death she has an I fraction. The only conclusion possible seems to be that under the conditions of this investigation (*i.e.*, no insistence on the patient's fasting prior to the collection of the blood) no importance whatsoever can be attached to Cronin Lowe's fraction.

The only other available series of follow-up cases is that recorded by Price in his University of Bristol Thesis of 1938 (4). He records a series of 30 cases. He, too, found the fraction so variable that it could not be used as a guide to the patient's progress. Six of his cases are not relevant to this discussion because they deal with innocent or untreated cases. There is no histological confirmation for several of his cases and only 2 of the cases were observed for longer than one year following treatment. These 2 cases were both rodent ulcers of the face. These came under his observation for the first time 26 and 48 months respectively after treatment had been commenced. The former had 2 tests with 3 months between them. Each of these gave initial flocculation of 27 and the patient was well when seen for the last time 6 months after the second test. The second case also had 2 tests, with 5 months between them. The first gave an initial flocculation of 27, and the second, one of 23. Further treatment had been given between the two tests and the patient was well when seen for the last time 3 months after the second test.

There are 18 cases in which the initial flocculation was variable or high in the tests performed during the first year after treatment, and, as already stated, these must be interpreted with caution.

There remain 4 cases in which the initial flocculation was persistently low during the first 12 months. They were observed for periods varying from 8 to 13 months. Three of these were only seen on one occasion 3 months after the last reading; 2 of them were then clinically well, while the third was clinically worse. The fourth case required further excision of the growth at the time of the second test and was then only seen once more, 3 months later.

It would appear to be fair to say that, within the short span of time during which Price's cases were under observation, the results roughly agree with those

here recorded. Real comparison is, however, impossible because, in his series, the time of observation is too short.

PRETREATMENT COLLOIDAL VANADATE REACTIONS

Samples of blood were collected from 65 patients before treatment was commenced and the results are shown in Table I. In this table, I is used to denote an innocent or indifferent fraction and M a malignant one.

When this table is analyzed on the assumption that readings above 25 are in the normal zone it shows that of the 38 patients who had an initial flocculation of 25 or over, 15 (39 per cent) are alive without recurrence, whereas of the 27 patients who had an initial flocculation of less than 25, only 4 are alive (16 per cent). This difference in proportion is roughly two and a half times its standard error and the first group may therefore be taken as having in all probability a significantly higher survival rate than the second.

Among the 20 patients who are alive (one of whom is known to have a recurrence) 11, or 55 per cent, had an innocent or indifferent fraction, and among the 45 who have died 23, or 51 per cent, had such a fraction.

These observations suggest:—1. That for a patient who has an initial flocculation of 25 or over before treatment there is a much better prognosis than for one in whom it is below 25. 2. That, under the conditions of this investigation (no insistence on the collection of blood in a fasting state), the fraction of the C.V.R. is no guide to prognosis. 3. That the fraction of the C.V.R. has no diagnostic value in random samples of blood.

TABLE I: PRETREATMENT COLLOIDAL VANADATE REACTIONS AND RESULTS

	Alive				Died			
Initial flocculation	Total	I	M	Average life, in months	Total	I	M	Average time to death, in months
28	..	..	..	..	1	1	.	20
27.5	..	..	..	..	1	..	1	4
27	2	1	1	63	3	1	2	24.5
26.5	1	1	..	44	1	..	1	28
26	5	3	2	51	7	3	4	23
25.5	5 *	2	3	44.5	6 †	2	4	18
25	3	1	2	70	3	2	1	24
24.5	1	..	1	56	8	7	1	19.5
24	..	..	..	..	7	2	5	23
23.5	1	1	..	56	1	1	..	5
23	2	2	..	46.5	3	2	1	14
22.5	..	..	..	..	..	..	..	..
22	..	..	..	..	1	..	1	3
21.5	..	..	..	..	2	1	1	10
21	..	..	..	..	1	1	..	11
Totals	20	11	9		45	23	22	

* 1 with known recurrence at 33 months.
† 1 committed suicide at 10 months

SUMMARY AND CONCLUSIONS

1. The results of the colloidal vanadate reaction (Bendien reaction) are presented for a series of proved cases of carcinoma of the cervix. All the patients were treated by radium except one which was treated by Wertheim's hysterectomy.

2. The results are analyzed in 2 groups and 2 types of observation are recorded in each group.

Group I. Repeated observations during the follow-up period (44 patients) for a. initial flocculation; b. fraction.

Group II. Pretreatment test (65 patients) for a. initial flocculation; b. fraction.

3. Analysis of the C.V.R. results in Group 1 above, taken in conjunction with the clinical course of the patient shows that:

a. (i) A graphic record of repeated initial flocculation estimations which remains practically horizontal is of good prognostic value and is found in most cases which clinically appear to be progressing satisfactorily.

(ii) An initial flocculation reading which falls within the zone 25 to 28, in cases which appear to be doing well, may be regarded as satisfactory and of good prognosis.

(iii) Graphic records of repeated initial flocculation estimations which show a falling curve are of serious prognostic import, particularly so if the patient appears clinically to be doing badly.

(iv) Initial flocculation values below 25 and particularly readings of 23 or less are indicative of bad prognosis and will be found

chiefly among those whose clinical progress is unsatisfactory.

b. The fraction is too variable (under the conditions of this investigation) to be of any value in prognosis.

4. Similar analysis of the Group II results shows:

a. The survival rate is significantly higher among those patients whose pretreatment initial flocculation is 25 or higher than in those in whom it is below 25.

b. Under the conditions of this investigation the pretreatment fraction is no guide to diagnosis or prognosis.

5. Cronin Lowe's "fraction," which was claimed to be of diagnostic value, has not been adequately tested in this series, insofar as no pre-test fasting has been demanded, but there is no doubt that the results show that it is unjustifiable to expect a pathologist to undertake the time-consuming laboratory investigations involved in assessing this "fraction" on blood samples collected under these conditions.

The author desires to record his deep debt of gratitude to Dr. Cronin Lowe for the immense amount of voluntary work he has put into the tests herein recorded, and for his patience in waiting some eight years for an independent assessment of their value. Gratitude is also expressed to Professor T. B. Davie who has given much time and care to reading the drafts of this paper and advising on its form and presentation.

REFERENCES

1. Bendien, S. G. T. Spezifische veränderungen Blutserums; ein Beitrag zur serologischen Diagnose von Krebs and Tuberkulose. Gustav Fischer, Jena, 1931. Translated into English by A. Piney. Specific Changes in the Blood Serum. A Contribution to the Serological Diagnosis of Cancer. Wm. Heinemann, Ltd., London, 1931.
2. Cronin Lowe, E. A Quantitative Modification of the Bendien Reaction in Serodiagnosis of Malignancy. Brit. M. J., **1**: 407-411. 1933.
3. Oliver, J. O. Sero-Diagnosis of Cancer and Experience with Modified Bendien Test. St. Thomas's Hosp. Rep., **2**: 5-16. 1937.
4. Price, C. H. G. The Three-Phase Modified Bendien Reaction. Brit. M. J., **1**:551-554. 1939.
5. Todd, T. F. Rectal Ulceration Following Irradiation Treatment of Carcinoma of the Cervix Uteri. Pseudo-Carcinoma of the Rectum. Surg., Gynec., & Obst., **67**:617-631. 1938.

Conservation of Scholarly Journals

The American Library Association created this last year the Committee on Aid to Libraries in War Areas, headed by John R. Russell, the Librarian of the University of Rochester. The Committee is faced with numerous serious problems and hopes that American scholars and scientists will be of considerable aid in the solution of one of these problems.

One of the most difficult tasks in library reconstruction after the first World War was that of completing foreign institutional sets of American scholarly, scientific, and technical periodicals. The attempt to avoid a duplication of that situation is now the concern of the Committee.

Many sets of journals will be broken by the financial inability of the institutions to renew subscriptions. As far as possible they will be completed from a stock of periodicals being purchased by the Committee. Many more will have been broken through mail difficulties and loss of shipments, while still other sets will have disappeared in the destruction of libraries. The size of the eventual demand is impossible to estimate, but requests received by the Committee already give evidence that it will be enormous.

With an imminent paper shortage attempts are being made to collect old periodicals for pulp. Fearing this possible reduction in the already limited supply of scholarly and scientific journals, the Committee hopes to enlist the cooperation of subscribers to this journal in preventing the sacrifice of this type of material to the pulp demand. It is scarcely necessary to mention the appreciation of foreign institutions and scholars for this activity.

Questions concerning the project or concerning the value of particular periodicals to the project should be directed to Wayne M. Hartwell, Executive Assistant to the Committee on Aid to Libraries in War Areas, Rush Rhees Library, University of Rochester, Rochester, New York.

Abstracts

Reports of Experimental Research

Carcinogenic Compounds

FALIN, L., and V. ANISSIMOVA. [Lab. of Histology, Medical and Stomatological Inst., Smolensk, Russia] TERATOÏDE DE LA GLAND SEMINALE DU COQ CAUSÉE PAR L'INJECTION D'UNE SOLUTION DE SULFATE DE CUIVRE. [TERATOMA OF THE SEMINAL GLAND OF THE COCK PRODUCED BY INJECTION OF COPPER SULFATE.] Bull. biol. et méd. expér. URSS, 9:518-520. 1940.

The authors injected 0.15 cc. of a 5 per cent copper sulfate solution into each genital gland of a cock. Autopsy 74 days later disclosed a tumor of the left genital gland weighing 79 gm. Microscopically this proved to be a teratoma, with a connective tissue stroma and rich in fat. Areas of hyaline and ossifying cartilage, and cystic cavities lined with cylindrical epithelium and surrounded with layers of smooth muscle were abundant. No necrosis or hemorrhage was seen.

The theory is advanced that the action of the copper sulfate (and of zinc salts) is due to tissue destruction which liberates substances acting as evocators that bring about teratoma production.—M. B.

LARIONOW, L. TH., M. A. CHERTKOVA, and A. S. SAMOKHVALOVA. [Lab. of Cancer Research, Dept. Path. Anatomy and Exper. Oncology of the State Roentgen, Radium, and Cancer Inst., Leningrad, Russia] ALTERATIONS OF THE BIOLOGICAL PROPERTIES OF CELLS IN TISSUE CULTURES UNDER THE ACTION OF CANCEROGENEOUS SUBSTANCES. Bull. biol. et méd. expér. URSS, 9:515-517. 1940.

The femoral muscle of newborn mice was cultured in a nutrient medium consisting of a mixture (1:1) of chicken and rabbit plasma and of chicken embryo extract. The liquid phase consisted of diluted chick embryo extract. Benzpyrene or dibenzanthracene was added to the culture medium in the form of a fine suspension prepared after the method of Boyland. Introduction of the carcinogen was begun from the onset of cultivation and usually continued uninterruptedly, although occasionally discontinued after varying intervals. Benzpyrene, in concentrations of 1 to 5 mgm. per cent was toxic, although dibenzanthracene did not display this effect.

In one series, a culture exposed to dibenzanthracene for 17 days began, 40 days after initial exposure, to grow very rapidly. Benzpyrene, even in high concentration, had no toxic or other effect upon its growth. Yet such cultures inoculated subcutaneously or intra-abdominally gave no growth.

Special secondary centers of growth were observed. These were formed by rapidly proliferating cells emerging from the periphery and swiftly outgrowing it. Cells from such centers exhibited greater mobility and a capacity for growth while isolated from each other. They contained large droplets of fat, were larger, and grew in more disorderly fashion than normal cultures. Far fewer of these cells were sufficient to start a subculture than is the case with untreated fibroblasts.

The supposition is held that carcinogens act directly on cells, although the possibility of other mechanisms cannot be excluded.—M. B.

MacKENZIE, I., and P. ROUS. [The Rockefeller Inst. for Med. Research, New York, N. Y.] THE EXPERIMENTAL DISCLOSURE OF LATENT NEOPLASTIC CHANGES IN TARRED SKIN. J. Exper. Med., 73:391-416. 1941.

Experiments were designed to prove that tarring renders many more cells neoplastic than ever assert themselves by forming tumors. However, no evidence was obtained that the cells which tar renders neoplastic respond in this manner because they are possessed of peculiarities not shared by the rest of the normal epithelium.

The skin of rabbits was tarred for a period shorter than that required to elicit growths and then subjected to noncarcinogenic stimulation, wound healing. It was found that though practically no growths appeared after a short period of tarring, tumors appeared on the healing surfaces of wounds inflicted after the initial tarring. Also, the second application of tar to the skin, after healing was completed, evoked many new tumors and they were more than three times as numerous in the areas where healing had taken place. The subject of tumor inception and tumor formation is discussed and in the light of the experimental findings "traumatic cancer" is explained. The authors suggest that the cancer formed after a blow or other injury may be due to the fact that the area may have contained many latent neoplastic cells which only manifest themselves by multiplying into growths after the noncarcinogenic stimulation of the injury.—D. S.

NEWMAN, M. S., and P. H. WISE. [Ohio State Univ., Columbus, Ohio] THE SYNTHESIS OF 5-METHOXY-10-METHYL-1,2-BENZANTHRACENE AND RELATED COMPOUNDS. J. Am. Chem. Soc., 63:2109-2111. 1941.

It is recorded that 5-methoxy-10-methyl-1,2-benzanthracene showed no carcinogenic activity within 10 months when tested by subcutaneous injection in mice.—H. J. C.

ROUS, P., and J. G. KIDD. [The Rockefeller Inst. for Med. Research, New York, N. Y.] CONDITIONAL NEOPLASMS AND SUBTHRESHOLD NEOPLASTIC STATES. A STUDY OF THE TAR TUMORS OF RABBITS. J. Exper. Med., 73:365-390. 1941.

After taking into consideration the characteristics of tumors, the authors come to the conclusion that "warts" (papillomas, carcinomatoids, frill horns) which are elicited by tar on rabbit skin are true tumors. These warts, however, are wholly dependent on aid for survival, for discontinuance of the tar applications causes retrogression of the papillomas and carcinomatoids and the epithelium may take on the appearance of ordinary epidermis. On the resumption of tarrings, warts are produced in greater abundance, earlier, and at the spots where previous ones had vanished. Also the recurrence of the warts was brought about by noncarcinogenic influences, turpentining and wound healing. The authors suggest that even though

tar warts regress and may take on the appearance of ordinary epidermis, the epithelium may retain its neoplastic potentialities for several months. They have reason to suppose, however, that in the end these tumor cells, unless helped, die or are cast off. The hypotheses that tumors are due to somatic mutations and to viruses respectively are discussed in the light of these phenomena.—D. S.

Viruses

DOERR, R. [Basel, Switzerland] DIE INFEKTION ALS GAST-WIRT-BEZIEHUNG MIT BESONDERER BERÜCKSICHTIGUNG DER TIERPATHOGENEN VIRUSARTEN. [INFECTION AS A HOST-PARASITE RELATIONSHIP WITH SPECIAL REFERENCE TO VIRUSES PATHOGENIC FOR ANIMALS.] Arch. f. d. ges. Virusforsch., 2:88-155. 1941.

In this extensive review, section VII, entitled "Symbiosis and Infection," contains a critical discussion of the relationship between latent infection of normal tissues and oncogenic viruses (pp. 131-134). The work of Rous, Andrewes, Doerr, and others is reviewed. No additional experimental material is presented on the problem of the possible relationship between viruses and tumors.—S. B-J.

FRIEDEWALD, W. F., and R. S. ANDERSON. [The Rockefeller Inst. for Med. Research and Memorial Hosp., New York, N. Y.] INFLUENCE OF EXTRANEOUS PROTEIN AND VIRUS CONCENTRATION OF THE INACTIVATION OF THE RABBIT PAPILLOMA VIRUS BY X-RAYS. J. Exper. Med., 74:463-487. 1941.

It was shown that the amount of irradiation required to inactivate the papilloma virus was not a fixed quantity, but was influenced by the concentration of virus in suspension and by the presence of extraneous protein in the virus preparation. While 2 to 4 million r were required to inactivate a virus preparation, only 50,000 to 400,000 r were needed to inactivate a similar preparation which was purified by differential centrifugation. Virus preparations purified by differential centrifugation were more resistant to x-rays, when suspended in the supernatant fluid from which they were obtained, or when suspended in normal rabbit serum or in a solution of crystalline egg albumen, than when they were suspended in saline. It was also found that the percentage inactivation of papilloma virus in purified suspensions increases as the concentration of virus is decreased by dilution. The effects of irradiation on serum antiviral antibody were also influenced by the presence of impurities. While 500,000 r had only a slight effect on the complement-binding antibody of the whole serum, the same radiation on the globulin fraction, which contained as high a titer of antibody as the original serum, was sufficient to inactivate it.—D. S.

KIDD, J. G. [The Rockefeller Inst. for Med. Research, New York, N. Y.] THE DETECTION OF A "MASKED" VIRUS (THE SHOPE PAPILLOMA VIRUS) BY MEANS OF IMMUNIZATION. RESULTS OF IMMUNIZATION WITH MIXTURES CONTAINING VIRUS AND ANTIBODY. J. Exper. Med., 74:321-344. 1941.

Experiments to answer the question, whether virus may be absent from some papillomas, or merely that its antigenicity as well as its pathogenicity may be somehow "masked" when the growths are extracted, were attempted. It was found: 1) that the antigenicity of virus filtrates was reduced or completely abolished when mixed with an excess of immune serum *in vitro;* 2) that resistance to the papilloma virus may be conferred by passively transferred antibody; 3) that a suspension of domestic rabbit papillomas that contained much extravasated antibody was wholly or almost wholly nonantigenic upon injection into normal rabbits and conferred no more resistance than could be accounted for by the passive transfer of the antibody contained in it; 4) that a suspension of domestic rabbit papillomas which contained little or no extravasated antibody and a small amount of infectious virus was antigenic, though not so much as a filtrate of wild rabbit papillomas that contained virus in abundance; 5) that extravasated antiviral antibody from papillomas reduced or abolished the antigenicity of the virus as did serum antibody; 6) and finally, that extracts of wild rabbit papillomas, even when they contained little or no infectious virus, have invariably proved more highly antigenic than those of domestic rabbit papillomas.—D. S.

NEURATH, H., G. R. COOPER, D. G. SHARP, A. R. TAYLOR, D. BEARD, and J. W. BEARD. [Duke Univ. Sch. of Med., Durham, N. C.] MOLECULAR SIZE, SHAPE AND HOMOGENEITY OF THE RABBIT PAPILLOMA VIRUS PROTEIN. J. Biol. Chem., 140:293-306. 1941.

The virus protein occurring naturally in warts in western cottontail rabbits was purified by processes of centrifugation. The preparations were studied by sedimentation velocity, diffusion, and viscosity methods, and it was found that the virus protein appeared to consist of only one molecular species with regard to size and shape. Electrophoretic measurements indicated that the protein is electrically homogeneous (see also, J. Biol. Chem., **142**:193. 1942). Biological studies on infectivity and neutralization with a specific immune serum also indicated the uniformity of the purified virus protein. The molecular weight is calculated to be 47,100,000 and the protein molecule resembles an oblate ellipsoid with an axial ratio of about 11 if hydration is neglected or of 7 if 33 per cent hydration is assumed.—H. J. C.

PACKALÉN, T. [Sch. of Hygiene and Public Health, Johns Hopkins Univ., Baltimore, Md.] A STUDY OF QUANTITATIVE CHANGES OF THE SHOPE RABBIT PAPILLOMA VIRUS AT THE SITE OF INOCULATION IN THE SKIN OF THE COTTONTAIL AND DOMESTIC RABBIT. J. Exper. Med., 73:1-5. 1941.

This preliminary study was carried out in order to find out: (a) the rate at which the inoculated virus disappears or is inactivated at the site of inoculation in the skin of the domestic rabbit; (b) whether the active virus persists at the site of inoculation during the whole incubation period in the cottontail rabbit or disappears for a time only to reappear in the developing papillomas. An extract of papillomas of cottontail rabbits was injected intracutaneously into 3 domestic and 2 cottontail rabbits. After varying intervals of time, the skin around the wheal was excised and extracted and the extract tested for virus activity by inoculation into domestic rabbits. It was found that the amount of active virus, at the site of inoculation, decreased markedly in the first hour and disappeared entirely after several hours in both the domestic and cottontail rabbit. The author believes that the rapid decrease and final disappearance of the virus is due to inactivation *in loco* and considers it unlikely to be due to the transportation of the virus away from the site of inoculation.—D. S.

ROUS, P., and W. F. FRIEDEWALD [Rockefeller Inst. for Medical Research, New York, N. Y.] THE CARCINOGENIC EFFECT OF METHYLCHOLANTHRENE AND OF TAR ON RABBIT PAPILLOMAS DUE TO A VIRUS. Science, 94:495-496. 1941.

Two groups of domestic rabbits were inoculated with papilloma virus by rubbing into 4 to 6 scarified areas of skin. After healing, tar and 0.3 per cent methylcholanthrene (in ether containing 2 per cent mineral oil) were applied to some of the inoculated areas, while others on the same animals were painted with the solvent, or a mixture of turpentine and acetone. Untreated papillomatous areas served as further controls. Applications were made 3 times weekly for 2 to 4½ months.

No cancer developed from the untreated papillomas or from those treated with the solvents. Malignant changes frequently occurred in the papillomas to which tar, and especially methylcholanthrene, was applied. The cancers derived directly from the virus-infected cells.

The possible role and relative importance of virus and carcinogen in producing malignancy are discussed.—M. B.

SYVERTON, J. T., R. A. HARVEY, G. P. BERRY, and S. L. WARREN. [Univ. of Rochester, Sch. of Med. and Dentistry, Rochester, N. Y.] THE ROENTGEN RADIATION OF PAPILLOMA VIRUS (SHOPE). I. THE EFFECT OF X-RAYS UPON PAPILLOMAS ON DOMESTIC RABBITS. J. Exper. Med., 73:243-248. 1941.

This report deals with the effects of roentgen radiation *in vivo* on papillomas that have been induced on domestic rabbits by the Shope papilloma virus. Papillomas on the ears of rabbits were exposed to single massive doses of x-rays ranging from 250 to 5,000 r. It was found that a single irradiation of less than 2,000 r failed to eradicate a single tumor, while dosages of 3,000 r effected the disappearance of 60% of the papillomas irradiated. A single dose of 3,500 r or higher "cured" 100% of the irradiated papillomas. On comparing the efficacy of a single large dose of x-rays with that of divided doses of 600 r each, given daily, it was found that the effects of fractional dosage approximate those of the single massive dosage. A total dosage of 3,000 r, applied fractionally "cured" 50% of the tumors irradiated, while a dosage of 3,600 r was uniformly effective. The authors believe that the effective elimination of papillomas from domestic rabbits by x-ray is due to detrimental effects on the rabbit's cells and not to detrimental effects on the virus, for they have shown that 14,000,000 r is necessary to destroy this virus.—D. S.

SYVERTON, J. T., G. P. BERRY, and S. L. WARREN. [Univ. of Rochester, Sch. of Med. and Dentistry, Rochester, N. Y.] THE ROENTGEN RADIATION OF PAPILLOMA VIRUS (SHOPE). II. THE EFFECT OF X-RAYS UPON PAPILLOMA VIRUS IN VITRO. J. Exper. Med., 74:223-234. 1941.

The effect of roentgen radiation *in vitro* on cell-free suspensions of papilloma virus, derived from cottontail rabbits, was studied in order to determine the dosage necessary to render papilloma virus noninfectious for both cottontail and domestic rabbits. Dosages of 10,000 to 2,000,000 r had no apparent effect on the virus. Irradiations of 2,000,000 to 14,000,000 r had an increasing effect on the virus; namely, a decrease in titer, lengthening of time of incubation and a decrease in size of papillomas. Above 14,000,000 r no papillomas could be induced.—D. S.

Biochemistry and Nutrition—Chemotherapy

AIVASIAN, A. I. [Lab. of Pathological Physiol. of the I. P. Pavlov 1st Med. Inst., Leningrad, Russia] CHANGES IN THE MINERAL METABOLISM ACCOMPANYING THE DEVELOPMENT OF EXPERIMENTAL CANCER. Bull. biol. et méd. expér. URSS, 9:521-523. 1940.

3,4-Benzpyrene (0.5% in benzene) was applied to the skin of the backs of 80 white mice. Twenty mice were similarly treated with benzene alone; 40 mice served as untreated controls. One drop was applied once every 3 days for 4 months; treatment was then discontinued. Two hours after the morning feeding (oats, milk) the urine was collected and a sample from the pooled output of 20 mice was tested for chlorides, sulfates, phosphates, and ether-sulfuric acids.

Application of benzpyrene or benzene had no effect on chloride output. A decrease became apparent when papillomas appeared and this became more pronounced with onset of carcinoma (gross diagnosis). It was also found that water output paralleled that of the chlorides.

Benzpyrene and benzene painting both lowered the output of phosphate and sulfate. With the appearance of papillomas the excretion of these substances increased to a level greater than those of the controls and further increases took place with onset of malignancy (phosphate 50%, sulfate 60%).

Neither benzene nor carcinogen application affected the output of ether-sulfuric acids. These increased at the papilloma stage and in animals with malignancy were twice above the normal output.—M. B.

DEUTSCH, H. F., B. E. KLINE, and H. P. RUSCH [Med. Sch., Univ. of Wisconsin, Madison, Wis.] THE OXIDATION OF PHOSPHOLIPIDS IN THE PRESENCE OF ASCORBIC ACID AND CARCINOGENIC CHEMICALS. J. Biol. Chem., 141:529-538. 1941.

Phospholipid oxidation was found to be catalyzed by the presence of ascorbic acid at pH 4 and this catalyzed oxidation was inhibited by the presence of benzpyrene, methylcholanthrene, dibenzanthracene, aminoazotoluene, and by hydroquinone. The importance of this inhibitory action in the mechanism of cancer formation is unknown.—H. J. C.

HAVEN, F. L., and S. R. LEVY [Sch. of Med. and Dentistry, Univ. of Rochester, Rochester, N. Y.] THE OCCURRENCE AND RATE OF TURNOVER OF TUMOR SPHINGOMYELIN. J. Biol. Chem., 141:417-425. 1941.

Sphingomyelin is present in rat carcinosarcoma 256 to the extent of about 0.3% of the wet weight. A higher phosphorus content was observed in the sphingomyelin isolated from the periphery of the tumor. As a result of experiments on the incorporation of radioactive phosphorus into tumor sphingomyelin, it is concluded that tumor sphingomyelin plays as important a role in phospholipid phosphorus metabolism as do lecithin and cephalin.—H. J. C.

SINAI, A. J. [Pathophysiological Lab., Central Inst. of Oncology, Moscow] DER GEWEBSSTOFFWECHSEL SARKOMKRANKER TIERE [METABOLISM CHANGES IN SARCOMA-BEARING ANIMALS.] Bull. biol. et méd. expér. URSS, 9:230-233. 1940.

Respiration and anaerobic glycolysis were studied in liver, spleen, kidney, diaphragm, and tumor in animals bearing the following neoplasms: Ehrlich mouse sar-

coma, Jensen rat sarcoma, and the Kritschewsky-Sinelnikow rat sarcoma. Using the Warburg apparatus, respiration was measured in an atmosphere of pure oxygen and anaerobic glycolysis in a mixture of 95% nitrogen plus 5% carbon dioxide. In all the organs studied, respiration was found to diminish and anaerobic glycolysis was increased. The possible bearing of other pathologic processes on these findings is briefly discussed.—M. B.

ZAHL, P. A., and L. L. WATERS. [Memorial Hosp. and the Haskins Lab., New York, N. Y.] LOCALIZATION OF COLLOIDAL DYES IN ANIMAL TUMORS. Proc. Soc. Exper. Biol. & Med., 48:304-310. 1941.

Nine newly prepared dyes were tested as to gross tumor-localizing properties, and their behavior is briefly described. Detailed study was made on Evans blue and lithium carmine.

It was found that these dyes do not localize in tumor cells but in the stroma surrounding the tumor, or within the tumor at interfaces of viable and necrotic tissue. Also the kidney and the reticulo-endothelial systems of liver and spleen take up considerable quantities of dye. X-ray treatment prior to dye injection did not appreciably alter the amount of dye that localized in the tumor, as determined histologically.

The dye in the tumor is so located that if it were replaced by a radioactive substance, effective radiation of the tumor edge would result.—M. B.

Clinical and Pathological Reports

Heart

SOMOLINOS D'ARDOIS, G. [Mexico, D. F.] CONTRIBUCIÓN AL ESTUDIO DE LOS TUMORES DESARROLLADOS EN EL CORAZÓN. [CONTRIBUTION TO THE STUDY OF TUMORS DEVELOPED IN THE HEART.] Arch. latino am. de cardiol. y hemat., X, 1:1-42. 1940.

This is a description of 5 cases of neoplasia of the heart of which one is a primary and the others are secondary tumors. The author gives first a résumé of 80 cases of primary tumor of the heart, published from 1865 to 1936, describing in summary its clinical and anatomical characteristics. The distribution according to the sexes was 48 males, 22 females, and 11 of unknown sex. In the age groups from 0 to 10 years, there were 2 cases; from 11 to 20, 6 cases; from 21 to 30, 14 cases; from 31 to 40, 12 cases; from 41 to 50, 14 cases; from 51 to 60, 12 cases; from 61 to 70, 8 cases; and above 71 years, 3 cases; in 10 cases the age was not specified. In the cases presented by the author the primary tumor is thoroughly described with post-mortem findings and microscopic study of the lesions. This description is followed by a discussion on the origin, anatomical pathology, and symptomatology of such primary tumors.

There follows a description of the 4 secondary cases preceded by some considerations on its incidence (1 to 1.5 per 1,000 autopsies), its mode of spread, and localization. According to the evidence gathered by the author (180 cases of secondary tumors of the heart) any organ bearing a sarcomatous neoplasm can produce metastases in the heart. The literature quoted is very extensive and the article is illustrated.—M. D-R.

Bone and Bone Marrow

ALBRIGHT, F., C. H. BURNETT, O. COPE, and W. PARSON. [Harvard Med. Sch. and Massachusetts Gen. Hosp., Boston, Mass.] ACUTE ATROPHY OF BONE (OSTEOPOROSIS) SIMULATING HYPERPARATHYROIDISM. J. Clin. Endocrinol., 1:711-716. 1940.

Immobilization of a large part of the skeleton in the adolescent individual may result in hypercalcinemia associated with rapidly developing osteoporosis. The osteoporosis is presumed to result from disuse. The hypercalcinemia may be diagnosed erroneously as hyperparathyroidism. It may prove fatal because of renal damage or the sequelae of hypercalcinemia. One case of a 14-year-old boy is reported in some detail, and an abstract is given of a case in a 9-year-old boy.—J. B. H.

BADGLEY, C. E., and M. BATTS, JR. [Univ. of Michigan Sch. of Med., Ann Arbor, Mich.] OSTEOGENIC SARCOMA. Arch. Surg., 43:541-550. 1941.

In this analysis of 80 cases, the authors found that the outstanding symptom is pain of increasing severity, followed by swelling. Most of the lesions were found in the ends of long bones, 50% appearing in the region of the knee. Amputation is the treatment of choice. The mortality was 79%. The average period of survival was 41 months. There were 15 five-year survivals and 4 ten-year survivals. The longest period of survival was 14 years, 7 months. The prognosis is more favorable in patients over 20 years of age. In the extremities, the prognosis is worse for the more centrally placed lesions. The prognosis varies directly as the degree of differentiation of the lesion.—G. De B.

FARROW, J. H., and H. Q. WOODARD. [Memorial Hosp., New York, N. Y.] THE INFLUENCE OF ANDROGENIC AND ESTROGENIC SUBSTANCES ON THE SERUM CALCIUM IN CASES OF SKELETAL METASTASES FROM MAMMARY CANCER. J.A.M.A., 118:339-343. 1942.

A study of 130 cases of cancer metastatic to bone showed numerous spontaneous disturbances in the serum calcium levels, most frequently when the breast was the site of the primary tumor. In 3 cases injections of testosterone propionate were followed by a decided rise in the concentration of serum calcium and in the urine calcium, accompanied by evidence of increased activity of the bone lesions. In 2 of these cases similar changes also followed injections of estrone. Apparently it is this increased activity of the bone lesions that accounts for the rise in calcium in the serum and urine, and contraindicates the use of testosterone.—H. G. W.

HORWICH, I. D. [Hosp. for Ruptured and Crippled, New York, N. Y.] INFILTRATING SQUAMOUS CELL EPIDERMOID CARCINOMA INVOLVING THE OS CALCIS. Am. J. Surg., 55:166-172. 1942.

Report of development of carcinoma in an old osteomyelitic sinus.—H. G. W.

MEYERDING, H. W. [Mayo Clinic, Rochester, Minn.] BENIGN AND MALIGNANT GIANT-CELL TUMORS OF BONE. J. A. M. A., 117:1849-1854. 1941.

A study of the diagnosis and results of treatment based on 124 patients with giant cell tumors of bone seen in

the Mayo clinic in 25 years, of which 57 were men and 67 women, and the youngest patient was 10 years old, with a mean age of 31.6 years. In 101 the lesions were benign, and 23 or 19% malignant, with a 5-year survival rate of 97% for the benign and 65% for the malignant. In tumors that are apparently benign, malignant changes may develop in time after surgical procedures or irradiation, or after a long period. Surgical removal when possible offers a rapid cure and permits an accurate microscopic diagnosis in the majority of cases, but irradiation has become of increasing value and may be used alone in some cases or as a post-operative adjunct. The advantage of a microscopic examination far offsets any danger which may be ascribed to biopsy.—H. G. W.

RENDICH, R. A., and A. H. LEVY. [Kings County Hosp., Brooklyn, N. Y.] UNUSUAL METASTATIC BONE LESIONS. Am. J. Roentgenol., 46:343-350. 1941.

Six case histories of carcinoma metastatic to bone are presented. The roentgenographs of these cases differed from the customary centralized appearance of bone metastases in that the lesions were corticomedullary and showed newly formed radiating bony spicules extending into the adjacent soft tissues as well as periosteal reactive triangles. The presenting symptoms were often due to the metastatic lesions and roentgenographically the disease might easily be confused with primary bone tumor. Primary tumors in these six cases were in the lung (1), thyroid (1), prostate (1), probably gastrointestinal tract (2), and unknown (1).—C. E. D.

SNYDER, C. H. [Butterworth Hosp., Grand Rapids, Mich.] MALIGNANT SYNOVIOMA OF THE KNEE JOINT. Am. J. Surg., 55:67-70. 1942.

About 15 cases of synovioma have been reported in the knee joint and 5 lateral and posterior to the knee, to which a case is added without recurrence in 18 months—H. G. W.

WILLIS, R. A. [Depts. of Pathology of the Alfred Hosp., Prahran, and the Univ. of Melbourne, Melbourne, Australia] SOLITARY PLASMOCYTOMA OF BONE. J. Path. & Bact., 53:77-85. 1941.

A case is described in which autopsy revealed fatal compression of the spinal cord due to a solitary plasmocytoma of the second cervical vertebra. Previously reported examples of solitary plasmocytoma of bone are reviewed, and it is concluded that the condition is probably a distinct entity and not merely an early localized stage of myelomatosis.—A. H.

Leukemia, Lymphosarcoma, Hodgkin's Disease

CHARACHE, H. [Brooklyn Cancer Inst., Brooklyn, N. Y.] TUMORS IN ONE OF HOMOLOGOUS TWINS: HODGKIN'S DISEASE: OSTEOGENIC SARCOMA. Am. J. Roentgenol., 46:69-74. 1941.

The author cites the common belief that tumors in homologous twins are "similar, symmetrical, and synchronous." He reports 2 cases of Hodgkin's disease and one of osteogenic sarcoma each affecting only one of a pair of homologous twins. The other twin in each case has remained unaffected during follow-up periods of 4 years, 5½ years, and 8 years respectively.—C. E. D.

O'BRIEN, F. W. [Boston, Mass.] END-RESULTS IN IRRADIATED HODGKIN'S DISEASE. Am. J. Roentgenol., 46:80-86. 1941.

The pathogenesis, diagnosis, prognosis, clinical classification, and treatment of Hodgkin's disease is discussed. Few cases are favorable for surgery and most are treated by radiation according to one of several technics. Heavy or protracted radiation therapy carries the hazards of severe hematopoietic damage or pulmonary fibrosis. Treatment must be highly individualized but is generally given in small fractionated doses with fields limited to the site of obvious disease.

Of 116 cases referred to the Boston City Hospital as Hodgkin's disease for radiation treatment, 29 proved to be some other condition, illustrating the importance of biopsy. Sixty cases with biopsy and adequate follow-up, had the disease for an average of 20.5 months before treatment and lived an average of 19 months following treatment. The author finds no evidence in his own experience or in the reports of others that irradiation prolongs the average length of life in Hodgkin's disease. Life may be prolonged in isolated cases and discomfort is frequently relieved.—C. E. D.

SHULMAN, S. [St. Joseph's Hosp., Far Rockaway, N. Y.] PRIMARY LYMPHOSARCOMA OF THE JEJUNUM. Am. J. Roentgenol., 46:182-184. 1941.

A case of primary lymphosarcoma of the jejunum in a 37-year-old man is reported. The tumor involved 4 feet of jejunum. Surgical resection was followed by roentgen therapy to the remaining metastatic lymph nodes. The patient died 5 months later from peritonitis due to leakage at the site of intestinal anastomosis. No tumor tissue was found at autopsy.—C. E. D.

Adrenal

HAMBLEN, E. C., W. K. CUYLER, and M. BAPTIST. [Duke Univ. Sch. of Med. and Hosp., Durham, N. C.] URINARY EXCRETION OF 17-KETOSTEROIDS IN OVARIAN FAILURE. I. IN HIRSUTISM AND VIRILIZING SYNDROMES. J. Clin. Endocrinol., 1:763-771. 1941.

Data from the literature that pertains to this subject are mentioned without critical analysis. Using the Oesting method and the Oesting-Hellige colorimeter, the urinary 17-ketosteroids have been estimated in 34 women from 17 to 48 years of age who had slight hirsutism or more frank evidences of virilism. In contrast to previous workers these authors report moderately elevated titers of 17-ketosteroids in association with even simple hirsutism and a parallelism between the titers of 17-ketosteroids and the degree of hirsutism.—J. B. H.

HAMBLEN, E. C., W. K. CUYLER, and M. BAPTIST. [Duke Univ. Sch. of Med. and Hosp., Durham, N. C.] URINARY EXCRETION OF 17-KETOSTEROIDS IN OVARIAN FAILURE. IV. DURING THE CLIMACTERIC AND AFTER ARTIFICIAL MENOPAUSE. J. Clin. Endocrinol., 1:777-781. 1941.

Using the Oesting method and the Oesting-Hellige colorimeter, urinary 17-ketosteroids were estimated in 41 women in whom menses had ceased. The authors attempt to make broad generalizations and interpret the data to support the theory that adrenal hyperactivity (17-ketosteroid excretion held to be an index of this) results when female reproductive function fails.—J. B. H.

Book Reviews

THE PROBLEM OF TUMOURS: AN EXPERIMENTAL INVESTIGATION. By J. C. Mottram, M.B. Lond., H. K. Lewis & Co., Ltd., 136 Gower Street, London, W. C. 1. VIII + 92 pages; 33 illustrations and 9 tables. Price 7s. 6d. net.

Among the many chemical and physical agents that will elicit malignant growth no common property has yet been found. The other side of the problem, a reaction common to the cells acted upon, has been hardly touched, writes the author, and it is to this task that he dedicates his little volume.

Examination of paramecia showed that carcinogenic hydrocarbons, heat, cold, ultraviolet radiation, and acids all produced the same result: increased viscosity of the cytoplasm. Probably as a result of this, division time was increased with the consequent emergence of a few cells that were abnormal in size, shape, and the number of their nuclei. The deformed organisms could then be cultivated indefinitely in the absence of the injurious agent without loss of the capacity to reproduce their kind, though they gave rise to normal forms as well.

Comparison of these misshapen paramecia with the pleomorphic cells of tumors leads to the conclusion that the two may perhaps be equivalent, and the product of some common, irreversible cytoplasmic change.

In discussing the significance of his findings the author expresses the belief that gene mutations do not explain the origin of neoplasia, and that its cause is not necessarily a virus.

The experiments are thought to support the view that in transforming the normal cell to a tumor cell the carcinogenic agents work directly, rather than indirectly by setting up in the tissues fibrosis, ischemia, or what not. For here there is no tissue to be acted upon.

His experiments, says the author, also answer the question: why do cells that normally seldom divide, such as those of muscle and nerve, rarely form tumors whereas those that often divide commonly do form them? In these protozoa abnormality begins with an inhibition of fission, and thus without cell division it cannot occur.

It is thought that the punctate way in which tumors arise is also explained, for when cultures of paramecia are subjected to carcinogenic agents inhibited mitoses are found in only a few of the organisms, and of these few only a very small number go on to produce abnormal races.

The author anticipates criticism on the score that analogies between the reactions of protozoa and of metazoan cells are fallacious. To this he replies that when carcinogenic agents are found to induce proliferation in mouse skin, in paramecia, and in yeasts the analogy, instead of being fallacious, is valuable since it indicates action on basic living processes rather than on specialized parts. And when it is found that these agents produce abnormal and monstrous cells in vertebrates, ciliates, yeasts, sea-urchin eggs, and worms it is evident that the analogy is not only justifiable but fundamental.

The book contains a few misprints, as does almost any first edition, and K. A. Heiberg's name appears as Heilberg in both text and bibliography. Save for these minor blemishes it is a creditable performance, and every investigator of cancer will wish to read for himself the experiments and deductions of this veteran student of the disease.

To the reviewer the testimony most damaging to the author's "cytoplasmic hypothesis" is the statement that during attempts to cultivate pure races of paramecia, normal, or apparently normal, forms were not infrequently encountered. The more normal these individuals the faster did they grow and reproduce, until sometimes they overgrew the abnormals. A reversion to normal does not occur in cancer; the world would be a much happier one if it did.

WILLIAM H. WOGLOM

MULE SPINNERS CANCER. EPITHELIOMA OF THE SKIN IN COTTON SPINNERS. By E. M. Brockbank, H. K. Lewis & Co., Ltd., 136 Gower Street, London, W. C. 1. 1941.

The author, who has seen over 180 cases of carcinoma of the skin in mule spinners, has collected in this small monograph a number of data about this form of cancer which are taken chiefly from the Report of the Departmental Committee appointed to consider evidence as to the Occurrence of Epitheliomatous Ulceration among Mule Spinners, London 1926 (referred to inaccurately in this book as "Home Office Report") and from the reports of the Manchester Committee on Cancer. The work initiated by the latter Committee showed that, in mice, "the application of a mixture of equal parts of lanolin and olive oil to the unwashed skin, before the mineral oil, prevented the development of dermatitis and epithelioma." The length of time necessary to demonstrate the same degree of protection in man is of course uncertain. In England in the period from 1923 to 1940 the average annual number of cases of this form of cancer was 63, with 20 deaths; in 1940 these numbers were 41 and 20 respectively.

E. L. KENNAWAY